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Role of Nimrod receptors in the Drosophila melanogaster cellular immune response

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Abstract

The use of modern molecular biology tools and simple but powerful tractable model organisms such as *Drosophila* has contributed significantly to our recent advances in the innate immunity field, notably phagocytosis. Since 2001, many phagocytic transmembrane receptors, potential opsonins, and in some cases their ligands, have been discovered. Among them, the Nimrod family, which contains key phagocytic receptors and potential opsonins, deserves special interest as accumulating genetic and biochemical evidence points to their critical role in phagocytosis of both bacteria and apoptotic cells. In this PhD thesis we provided a deeper characterization of two Nimrod transmembrane receptors, Eater and NimC1. In particular, by using single or combined mutations in *eater* and *NimC1*, we aimed to further elucidate i) the involvement of each receptor in bacterial phagocytosis and ii) the role of Eater in hemocytes sessility and adhesion. Both receptors have been shown to be specifically expressed in hemocytes, the insect blood cells, and to mediate Gram-positive but not Gram-negative bacteria phagocytosis. In this work we generated a novel *NimC1* mutant and demonstrated that the single *NimC1* deletion does not affect phagocytosis of bacteria. However, by using the compound *NimC1;eater* mutant we were able to show that these receptors contribute in a synergistic way to microbes engulfment, but that Eater can bypass the requirement for NimC1. Moreover, we discovered that NimC1, but not Eater, is required for phagocytosis of non-immunogenic particles, namely latex beads and yeast zymosan. Therefore, this work provides evidences of Eater and NimC1 being the main receptors for bacteria phagocytosis, and suggests that those proteins likely play distinct roles in microbial uptake, as tethering and docking receptors. The second part of this thesis re-addresses the role of Eater in hemocyte sessility and adhesion. A previous study showed that this phagocytic receptor is essential to mediate blood cell sessility to the animal integument during larval stages. However, a series of questions about Eater-mediated sessility remains still open. For example, is Eater mediating hemocyte adhesion also in adult flies? And yet, does Eater mediate hemocyte sessility by regulating the expression of cell adhesion genes, therefore functioning as a signalling receptor? By using $eater^{1}$ mutant adult flies, we showed that, as in larval stages, Eater is required in adult hemocytes to confer adhesion and sessility. Interestingly, we uncovered a potential role of sessility in hemocyte survival during adulthood, since $eater^{1}$ flies have a decreased number of blood cells. Preliminary transcriptomics analysis led us to hypothesise that this receptor might not work as a signalling molecule, but rather as an adhesion protein providing the initial hemocyte contact

to its target surface. In line with Eater's role as an adhesion molecule, its over-expression in hemocytes leads to the formation of enlarged cells compared to control.
To conclude, this thesis extended our knowledge on the *Drosophila* cellular immune response, by providing new insights on two Nimrod phagocytic receptors.

Key words

Drosophila, innate immunity, phagocytosis, hemocytes, Nimrod, adhesion, proliferation, cell survival, sessility.

Riassunto

L'uso di moderne tecnologie nel campo della biologia molecolare e di potenti, ma semplici organismi modello come la *Drosophila* hanno contribuito in modo significativo ai nostri recenti progressi nel campo dell'immunità innata, in particolare la fagocitosi. A partire dal 2001, sono stati scoperti diversi recettori transmembrana e potenziali opsonine, nonché, in alcuni casi, anche i loro ligandi. Tra questi, la famiglia Nimrod merita un interesse in particolare in seguito all'accumulo di molteplici prove genetiche e biochimiche che sembrerebbero evidenziare il loro ruolo nella fagocitosi di batteri e cellule apoptotiche. In questa tesi di dottorato abbiamo fornito una caratterizzazione più approfondita di due recettori transmembrana Nimrod, Eater e NimC1. In particolare, usando mutazioni singole o combinate in *eater* e *NimC1*, abbiamo chiarito ulteriormente i) il coinvolgimento di ciascun recettore nella fagocitosi batterica e ii) il ruolo di Eater nella sessilità e adesione degli emociti. È stato precedentemente dimostrato che entrambi questi recettori sono espressi specificamente negli emociti, e che contribuiscono alla fagocitosi di batteri Gram-positivi, ma non Gram-negativi. In questo lavoro abbiamo generato un nuovo mutante *NimC1* e dimostrato che la singola delezione in *NimC1* non influenza la fagocitosi batterica. Tuttavia, usando il doppio mutante *NimC1;eater* siamo stati in grado di dimostrare che questi recettori contribuiscono sinergicamente all'internalizzazione dei microbi, e che Eater può bypassare il requisito di NimC1. Inoltre, abbiamo scoperto che NimC1, ma non Eater, è necessario per la fagocitosi di particelle non immunogene, vale a dire latex beads e zymosan. Questo lavoro fornisce evidenze che Eater e NimC1 sono quindi i principali recettori della fagocitosi batterica e suggerisce che tali proteine svolgono probabilmente ruoli distinti nell'assorbimento microbico, come recettori di tethering e docking. La seconda parte di questa tesi riaffronta il ruolo di Eater nella sessilità e adesione degli emociti. Uno studio precedente ha dimostrato che questo recettore è essenziale nel mediare la sessilità delle cellule sanguigne al tegumento animale durante le fasi larvali. Tuttavia, diverse domande su come Eater media la sessilità negli emociti rimangono aperte. Ad esempio, Eater media l'adesione degli emociti anche nelle mosche adulte? Eater media la sessilità regolando l'espressione dei geni coinvolti nell'adesione cellulare, funzionando quindi come recettore di segnalazione? Usando mosche adulte mutanti per *eater*, abbiamo dimostrato che, come negli stadi larvali, Eater è richiesto negli emociti adulti per conferire aderenza e sessilità. Inoltre, abbiamo abbiamo scoperto un potenziale ruolo della sessilità nella sopravvivenza degli emociti durante l'età adulta, poiché le mosche *eater*^{1} hanno un numero ridotto di cellule sanguigne.

L'analisi preliminare di trascrittomica ci ha portato a ipotizzare che questo recettore potrebbe non funzionare come una molecola di segnalazione, ma piuttosto come una proteina di adesione che fornisce il contatto iniziale degli emociti con la loro superficie bersaglio. In linea con il ruolo di Eater come molecola di adesione, la sua sovraespressione negli emociti porta alla formazione di cellule più allargate rispetto al controllo.

Per concludere, questa tesi ha ampliato le nostre conoscenze sulla risposta immunitaria cellulare della *Drosophila*, fornendo nuove conoscenze sui recettori fagocitari Nimrod.

Parole chiavi

Drosophila, immunità innata, fagocitosi, emociti, Nimrod, adesione, proliferazione, sopravvivenza cellulare, sessilità.

Table of contents

Abbreviations

MAMP	Microbe associated molecular pattern
PRR	Pattern recognition receptor
SR	Scavenger receptor
PGRP	Peptidoglycan recognition protein
Nim	Nimrod
Hml	Hemolectin
Srp	Serpent
AMP	Antimicrobial peptide
PPO	Prophenoloxidase
CNS	central nervous system
PS	Phosphatidylserin

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 1: Introduction

1.1 The basis of *Drosophila* innate immunity

As all organisms, insects live in a microbe-rich environment. Furthermore, some insects feed themselves, lay eggs and develop on decomposing organic media, thus being significantly more exposed to invaders. In contrast to vertebrates which developed two different immune defence mechanisms, the adaptive and the innate one, arthropods completely rely on the latter. The backbone of innate immunity consists on the host ability to distinguish between self and foreign non-self (Janeway and Medzhitov, 2002). This recognition is mediated by the binding of specific host pattern recognition receptors (PRRs) to microbe-associated molecular patterns (MAMPs) (Akira et al., 2006). MAMPs are molecules produced by invading microorganisms which are normally not present in host cells. Examples of well characterized MAMPs are the bacterial cell wall component peptidoglycan (PGN) and the fungal cell wall component ß-1,3-glucan (Gillespie et al., 1997). Hence, the insect's immune system totally relies on PRRs in order to recognize invading pathogens and induce an immune response against them.

Thanks to its outstanding collection of genetic and genomic tools, as well as the remarkable conservation of immune pathways with higher organisms, *Drosophila* has been a powerful model to investigate innate immunity and host-pathogen interactions (Buchon et al., 2014; Lemaitre and Hoffmann, 2007). *Drosophila* lives in a rotting organic environment enriched in microorganisms. For this reason, it developed several strategies in order to recognize and fight infectious intruders. As a first line of defence, *Drosophila* uses epithelial barriers to prevent microorganisms from entering its body cavity. However, when microbes are able to overcome these initial physical obstacles, *Drosophila* can mount both cellular and humoral innate immune reactions in order to fight against invaders (Lemaitre and Hoffmann, 2007). While the humoral immune response involves the induction of antimicrobial peptides (AMPs) expression in the fat body (the *Drosophila* liver-analogue), the cellular immunity relies on specialized blood cells (or hemocytes), which participate in melanization responses, engulfment of bacteria, and encapsulation of intruders in the hemolymph. In order to accomplish those tasks, *Drosophila* relies on three main classes of differentiated hemocytes, each one having a specific immune function: plasmatocytes, crystal cells, and lamellocytes. These three cell types derive from a common undifferentiated progenitor, called prohemocyte (**Fig. 1**).

The following paragraphs will introduce the main components of the cellular immune branch, focusing later on the phagocytosis process, the main topic of this thesis.

1.2 *Drosophila* blood cell types

Plasmatocytes are professional phagocytes sharing functional features with mammalian macrophages, and represent the most abundant hemocyte class (90-95% of total hemocytes) at all developmental stages (Lanot et al., 2001). In addition to their phagocytic tasks (that will be discussed further later), plasmatocytes perform a number of physiologic functions during development. Embryonic plasmatocytes mediate the secretion of extracellular matrix (ECM) components such as Laminin, Tiggrin, Papilin, Collagen IV, Peroxidasin and ßPS integrin (Bhave et al., 2012; Comber et al., 2011; Fogerty et al., 1994; Kramerova et al., 2003; Kusche-gullberg et al., 1992; Martinek et al., 2008; Mirre et al., 1988; Nelson et al., 1994; Yasothornsrikul et al., 1997). The importance of plasmatocytes in tissue remodelling through the production of ECM-associated proteins has been proven for sculpting of the Malpighian-renal tubules (Bunt et al., 2010) and for central nervous system development (Olofsson and Page, 2005). Mature larval plasmatocytes also possess antimicrobial activity, and are involved in the first stages of encapsulation of parasitic intruders (Irving et al., 2005).

The remaining 5-10% of the blood cell population is represented by crystal cells, which are instead non-phagocytic cells involved in the production and deposition of melanin (Binggeli et al., 2014; Meister, 2004). Melanization is usually observed at the surface of invading parasites in the hemocoel and at the site of cuticular injury. The enzymes responsible for melanin biosynthesis are called prophenoloxidases (PPOs). PPOs are stored as inactive, crystalline structures in crystal cells, but upon cellular release they undergo activation through a proteolytic cascade mediated by serine proteases. Melanization represents a key process for successful wound healing and during encapsulation of parasites (Vlisidou and Wood, 2015). Moreover, it also participates in the sequestration of microorganisms at the site of injury and in their elimination through the production of toxic intermediates, such as peroxide and nitric oxide. *Drosophila* genome encodes for three PPOs: PPO1, PPO2, and PPO3 (Binggeli et al., 2014; Dudzic et al., 2019). While the latter is specifically expressed in lamellocytes, PPO1 and PPO2 are both expressed by crystal cells, being the main enzymes involved in melanization (Binggeli et al., 2014; Dudzic et al., 2019; Irving et al., 2005). As an evidence of the involvement of crystal cells and melanization in *Drosophila* innate immunity, *PPO1* and *PPO2* mutant larvae were shown to be more susceptible to bacterial infection (Binggeli et al., 2014;

Dudzic et al., 2019). Finally, several hemocytes mutants, either lacking blood cell populations or carrying aberrant crystal cells, showed impaired melanization at sites of injury, resulting in less-efficient wound healing and higher mortality (Galko and Krasnow, 2004; Neyen et al., 2015; Rämet et al., 2002; Rizki et al., 1980).

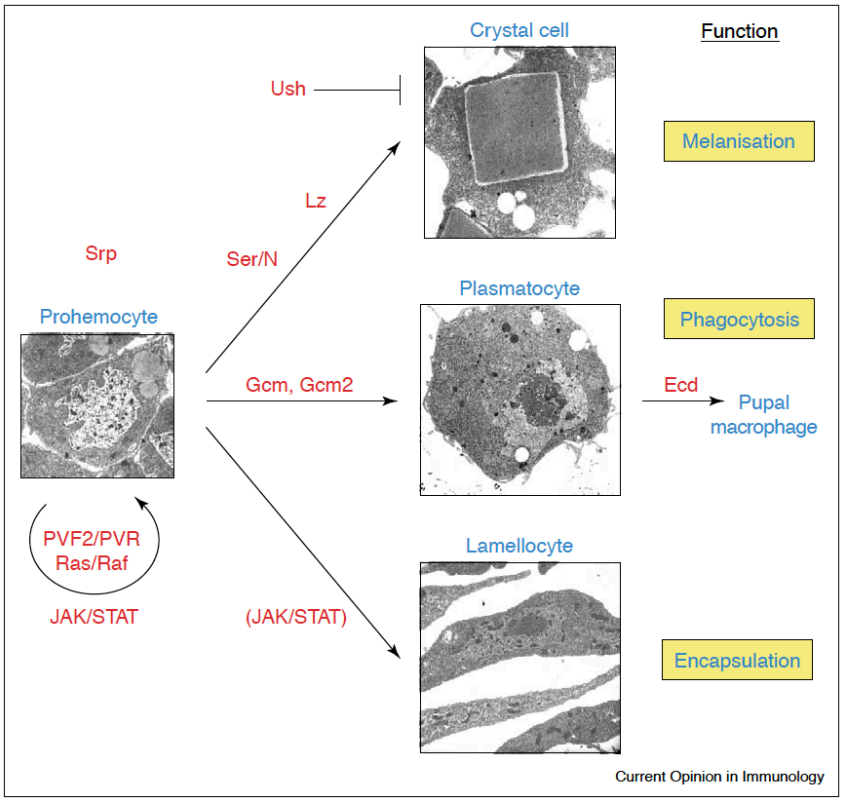

Figure 1. *Drosophila* blood cell types. *Drosophila* hemocytes originate from multipotent progenitors called prohemocytes, which can differentiate into three mature cell types: plasmatocytes, crystal cells and lamellocytes. Plasmatocytes are phagocytic cells and represent the most abundant hemocyte class at all developmental stages. Lamellocytes are barely present in healthy larvae, but can differentiate from plasmatocytes or prohemocytes in response to specific stress signals, such as parasitization. They are thought to play an essential role in encapsulation of parasitoid wasp eggs. The remaining 5% to 10% of the blood cell population is represented by crystal cells, which are non-phagocytic cells involved in the melanization response and wound healing. In red are shown the main pathways and/or factors responsible for hemocyte cell fate determination and proliferation (from Meister, 2004).

Lamellocytes are large, flat and highly adhesive cells rarely present in healthy larvae, but they can differentiate during larval stages upon specific stress signals such as parasitization, injury, or in tumorous larvae (Vlisidou and Wood, 2015). Two different types of lamellocytes can be observed in a *Drosophila* larva upon wasp parasitization (Anderl et al., 2016; Honti et al., 2010; Stofanko et al., 2010). A specific lineage (called "type II") arises by transdifferentiation of "activated plasmatocytes" which adhere to the surface of the wasp egg after its deposition in the larval hemocoel. These cells express markers for both mature plasmatocytes and

lamellocytes. The circulating "type I" lamellocytes, instead, are generated by a designated lamelloblast lineage, which is in turn induced from prohemocytes after infection. In contrast to the type II, these circulating lamellocytes do not express significant levels of plasmatocytes markers (Anderl et al., 2016). Several conditions have been reported to trigger lamellocytes differentiation in the absence of wasp egg deposition (Schmid et al., 2016; Stofanko et al., 2010; Zettervall et al., 2004).

1.1.2 Different origins of *Drosophila* hemocytes

Hemocytes development in *Drosophila* occurs into two spatially and temporally distinct waves, and can be classified into embryonic and larval (or lymph gland) hematopoiesis (**Fig. 2**) (Banerjee et al., 2019).

During the first hematopoietic wave, hemocytes progenitors originate from the embryonic procephalic mesoderm (**Fig. 2A**) (Holz, 2003; Tepass et al., 1994). These prohemocytes undergo four rounds of cell division before differentiating into mature phagocytic hemocytes and crystal cells. Hemocytes proliferation has been shown to be regulated by the PDGF/VEGF receptor (Pvr) and its ligand Pvf2, as well as by the Ras/Raf and the JAK/STAT pathways (**Fig. 1**) (Asha et al., 2003; Luo et al., 1995; Munier et al., 2002). By the end of embryogenesis, plasmatocytes have spread throughout the embryo, with crystal cells remaining mainly localized at their site of differentiation. Prohemocyte identity is specified by the GATA transcription factor Serpent (Srp), which is considered as the master regulator of *Drosophila* hematopoiesis (Abel et al., 1993; Sam et al., 1996). Glial cells missing (Gcm) and Gcm2 are then required for plasmatocyte differentiation. Crystal cells fate is instead specified by the Serrate/Notch pathway (Duvic et al., 2002; Lebestky et al., 2003) and by the transcription factor Lozange (Lz) (Lebestky et al., 2000). In addition, the Friend-of-GATA homolog U-shaped, whose expression is regulated by Srp, has been shown to act as a negative regulator of crystal cells development (**Fig. 1**) (Fossett et al., 2003; Fossett and Schulz, 2001; Muratoglu et al., 2006; Waltzer et al., 2003, 2002). During the following larval stages, the embryonic hemocytes population expands in number by self-renewal, colonizing specific local microenvironments. In the *Drosophila* larva, indeed, in addition to the lymph gland and hemolymph, hemocytes can also be found in a third compartment: the sessile hematopoietic tissues (or hematopoietic pockets) (Honti et al., 2010; Leitão and Sucena, 2015; Markus et al., 2009; Zettervall et al., 2004). Here hemocytes attach to the inner layer of the cuticle, forming a striped pattern along the dorsal vessel and lateral patches, in association with the endings of peripheral neurons and

oenocytes, a hepatocyte-like cells (Makhijani et al., 2011; Makhijani and Brückner, 2012). Increasing evidences suggest that this sub-epidermal sessile compartment of hemocytes function as an active hematopoietic site (Leitão and Sucena, 2015; Markus et al., 2009). In particular, it has been shown that peripheral neurons function as a niche, regulating hematopoiesis and hemocyte sessility. A key factor provided by peripheral neurons to the hematopoietic pockets is Activin-ß/TGF-ß, whose signalling promotes hemocytes proliferation and adhesion (Makhijani et al., 2011). In these sites, mature sessile plasmatocytes transdifferentiate into crystal cells, in a process dependent by Notch signalling (Leitão and Sucena, 2015). Moreover, upon wasp infestation, those sessile plasmatocytes can also transdifferentiate into lamellocytes (Markus et al., 2009), functioning as an early source of lamellocytes. Plasmatocytes sessility at these sites is disrupted in several classes of mutants and hemocytes release from the hematopoietic pockets is regulated by a series of pathways (such as ecdysone signalling at metamorphosis onset) (Bretscher et al., 2015; Makhijani et al., 2011; Regan et al., 2013; Schmid et al., 2016; Stofanko et al., 2010; Williams et al., 2006; Zettervall et al., 2004).

The second set of hemocytes derives from the lymph gland, a specialized hematopoietic organ that originates from the embryonic dorsal mesoderm (**Fig. 2B**) (Rugendorff et al., 1994). *Drosophila* lymph gland develops and becomes fully mature during the following larval stages, acting as a reservoir of both prohemocytes and mature hemocytes. Lymph gland structure is defined by an anterior pair of lobes (also called primary lobes), and a variable number of posterior lobes, which flank the dorsal vessel (the *Drosophila* heart-like organ). The posterior lobes mainly host quiescent prohemocytes during larval development, that will differentiate into mature hemocytes near the onset of metamorphosis. The primary lobes, instead, can be divided into three distinct and characteristic zones: the posterior signalling centre (PSC), the medullary zone (MZ), and the cortical zone (CZ) (Jung, 2005; Lebestky et al., 2003). The PSC function as a hematopoietic niche, supporting lymph gland hemocyte homeostasis. PSC cells are positive for several markers, including, Antennapodia, Collier and Hedgehog (Crozatier et al., 2004; Lebestky et al., 2003; Mandal et al., 2007). The MZ consists of hemocyte precursors and the CZ harbours differentiating hemocytes (i.e. plasmatocytes and crystal cells). MZ cells express Domeless (Dome, the upstream receptor of JAK/STAT pathway), Tep4 and E-Cadherin, whereas differentiated hemocytes from the CZ are positive for markers such as Lz, PPO1, PPO2, Peroxidasin (Pxn), Hemolectin (Hml), NimC1 antigen P1, or Eater (Binggeli et al., 2014; Jung, 2005; Kurucz et al., 2007a, 2007b; Lebestky et al., 2000; Makhijani et al., 2011; Rizki et al., 1980; Sinenko and Mathey-Prevot, 2004; Stofanko et al., 2010). In the rare

events that lamellocytes are detected within the CZ of unchallenged larvae, they can specifically be labelled by L1/Atilla, Misshapen (Msn), αPS4 integrin and Myospheroid (Mys) markers (Braun et al., 1997; Crozatier et al., 2004; Irving et al., 2005; Kurucz et al., 2007b; Markus et al., 2009; Tokusumi et al., 2009). Recent studies have revealed the presence of a pool of actively dividing intermediate progenitors positioned between the MZ and CZ, in the so called intermediate zone (IZ) (Krzemien et al., 2010; Sinenko and Mathey-Prevot, 2004). Those cells are characterized by the simultaneous expression of MZ markers (such as Dome) and the early hemocytes differentiation markers Hml and Pxn. However, intermediate progenitors are not positive for mature plasmatocytes (P1) and crystal cells (Lz) markers (Krzemien et al., 2010). The intermediate progenitors have been described as a population of actively dividing cells that has left the MZ, undergoing terminal division before differentiating (Krzemien et al., 2010).

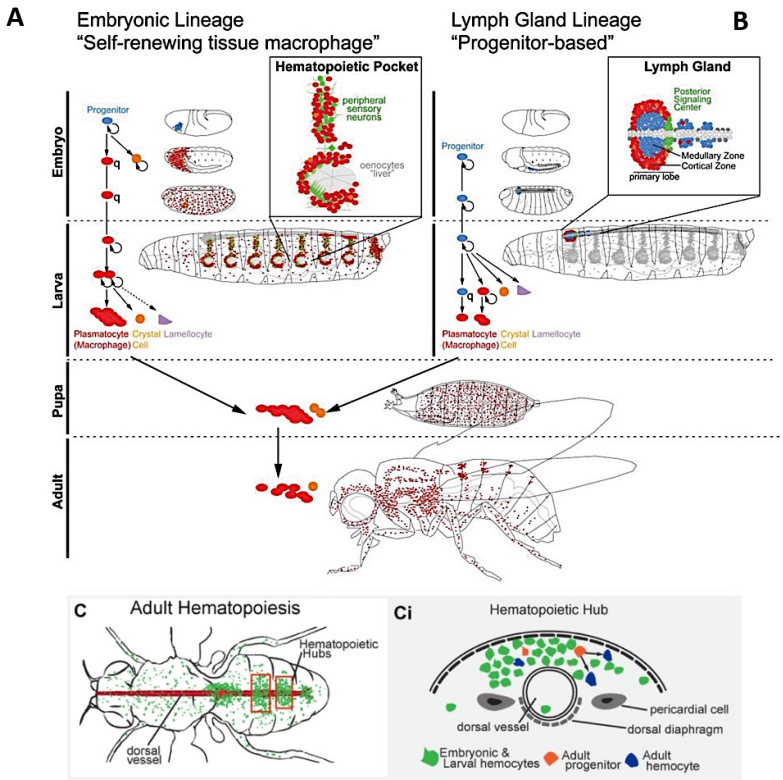

Figure 2. *Drosophila* hematopoiesis. (A) Schematic representation of embryonic hematopoiesis. This first wave of hematopoiesis generates a defined number of hemocytes (plasmatocytes and crystal cells). During larval stages, plasmatocytes colonize local microenvironments, notably the hematopoietic pockets, and proliferate through self-renewal. Here, plasmatocytes can transdifferentiate into lamellocytes, upon specific immune challenge, or crystal cells. (B) Lymph gland hematopoiesis. In a third instar larva, the fly lymph gland is fully mature, and can be subdivided into primary and secondary lobes. While the latter only harbours prohemocytes, the primary lobes are organized into a cortical zone of differentiated hemocytes, a medullary zone of quiescent blood cells progenitors, and a posterior signalling center, which act as a supportive hematopoietic nice. Hemocytes of both origins are mobilized during pupa and persist into the adult as a mixed population. (C) Adult hematopoiesis. Active hematopoietic progenitors are located in hematopoietic hubs (Ci). These blood cells can differentiate upon immune challenge (adapted from Gold and Brückner, 2015; Parsons and Foley, 2016).

During the transition to pupariation the lymph gland breaks to release the differentiated blood cells in circulation (a process promoted by the ecdysone steroid signalling (Sampson et al., 2013)), where they play crucial roles in tissue remodelling. Both blood cells lineages (embryonic and lymph gland) persist into the adult stage of the fly as a mixed population. Adult hematopoiesis in *Drosophila* has often been the subject of active debates. A recent work identified clusters of hemocytes located in abdomen hubs, which proliferate after bacterial infection (**Fig. 2C-Ci**) (Ghosh et al., 2015). These hematopoietic hubs also harbour hemocytes precursors of larval lymph gland origin, that differentiate into crystal cells and actively phagocytic plasmatocytes upon immune challenge. Nevertheless, several numbers of studies underlined the absence of active hematopoiesis in the adult stage, supporting the idea of hemocytes immunosenescence throughout adulthood (Bosch et al., 2019; Horn et al., 2014; Lanot et al., 2001; Van De Bor et al., 2015). In particular, a recent study did not report a significant increase in the total number of adult hemocytes neither during adult maturation, nor after septic injury, as a further proof for no active hematopoiesis in adult *Drosophila* flies (Bosch et al., 2019).

1.3 The phagocytosis process in *Drosophila*

Phagocytosis is defined as the cellular uptake of particles bigger than 0.5 μm through the formation of a membrane derived vesicle known as the phagosome. Its first description dates back to over 100 years ago, when the Russian zoologist Elie Metchnikoff observed that specific cells in starfish larvae were able to engulf foreign objects. This very simple observation turned out to be a crucial milestone in the history of cellular innate immunity. Phagocytosis is an ancient and evolutionarily conserved process performed by unicellular organisms and different metazoan cell types. In the former case, phagocytosis represents an important feeding mechanism (Cosson and Soldati, 2008). In higher organisms, instead, phagocytosis is mediated by dedicated cells, called phagocytes, which are able to digest both pathogens and "altered-self" particles. In this way, phagocytosis not only represents a crucial first line of defence but, importantly, also mediates tissue homeostasis via the clearance of apoptotic and necrotic cells. Despite major breakthroughs made after its discovery, the detailed molecular mechanisms underlying phagocytotic processes remain poorly understood. Mammalian systems are characterized by highly complex and redundant phagocytic components, which complicates addressing the specific role of each protein. Therefore, in the last decades, researchers have

expanded their studies of phagocytosis to genetically tractable model organisms, such as the fruit fly *Drosophila melanogaster* (*Drosophila*). *Drosophila* relies entirely on the innate immune response to fight infections (Lemaitre and Hoffmann, 2007). It has a less redundant genome, which has been fully sequenced and extensively annotated, and exhibits powerful genetic and molecular techniques. These reasons made *Drosophila* an attractive and suitable model system to study the complex process of phagocytosis. The following paragraphs will provide an up to date of phagocytosis in this insect. After describing the nature of the cells that are capable of phagocytosis, I will describe phagocytic receptors and opsonins that initiate the process of engulfment as well as the cellular machinery that internalizes particles and leads them to destruction. The second part of this introduction will analyse how phagocytosis contributes to development, maintenance, and host defence at the organismal level.

1.3.1 Professional and non-professional phagocytes in *Drosophila*

Different cell types have been reported to engulf foreign materials in *Drosophila*. In particular, we can distinguish between professional (globally called macrophages) and non-professional phagocytes (tissue-resident neighbouring cells). *Drosophila* possesses specialized hemocytes (i.e. blood cells) named plasmatocytes or macrophages that function as professional phagocytes. They engulf pathogens, apoptotic cells and dendrite debris. Non-professional phagocytes are usually tissue-resident cells, which in addition to their established tasks can also engulf foreign particles, in environments where circulating macrophages are less accessible. However, non-professional phagocytes frequently display decreased phagocytic abilities compared to macrophages, with respect to the variety and efficiency of particles they can take up. Nevertheless, the recognition and signalling machinery appear similar in both cell types. Several cell types have been shown to function as non-professional phagocytes in *Drosophila* (Shklover et al., 2015a), mainly mediating apoptotic cell clearance (a process also described as efferocytosis). Glial cells are one of the best characterized non-professional *Drosophila* phagocytes which mediate the shaping of the central nervous system (CNS) in developing embryos. Ovarian follicle epithelial cells are another well studied example of non-professional phagocytes. In particular, they mediate efferocytosis of nurse cells inside the egg chamber during *Drosophila* oogenesis (Serizier and McCall, 2017).

1.3.2 Phagocytic receptors and opsonins in *Drosophila*

Phagocytosis is usually initiated by dedicated receptors that bind to molecules exposed on the surface of pathogens or apoptotic cells. Well-established microbial associated molecular patterns (MAMPs) recognized by phagocytic receptors are bacterial peptidoglycans, lipopolysaccharides (LPS), or fungal ß-1,3 glucans. Apoptotic cells are often decorated by phosphatidylserine (PS), a membrane phospholipid species usually found in the inner leaflet of the plasma membrane, but exposed on the surface of cells undergoing apoptosis (Segawa and Nagata, 2015). Upon ligand recognition, phagocytic receptors directly or indirectly engage downstream signalling pathways that initiate the uptake of the particle. Studies have identified a plethora of receptors with a putative role in phagocytosis, based on their similarity with engulfment receptors in other species. While some may be true phagocytic receptors, others may indirectly affect phagocytosis or be involved in the downstream steps. In the following paragraphs, I provide a critical summary of the main phagocytic receptors and potential opsonin molecules functioning in bacterial phagocytosis and apoptotic cell clearance in *Drosophila* (**Fig. 3**).

1.3.2.1 Scavenger receptors

Scavenger receptors (SRs) are transmembrane proteins expressed by both invertebrate and mammalian professional phagocytes. Their activity was first identified in mammalian macrophages where they endocytose modified low-density lipoprotein (mLDL) (Brown and Goldstein, 1983). In addition, SRs are also able to bind to polyanionic ligands and function as pattern recognition receptors (PRRs), mediating phagocytosis of microbes and dying cells in many species. One of the first SRs discovered in *Drosophila* is class C Scavenger receptor I (dSr-CI). The class C Scavenger receptor family counts four members in *Drosophila* (dSr-CI, dSr-CII, dSr-CIII, dSr-CIV). dSr-CI is specifically expressed on plasmatocyte surfaces and was shown to possess a similarly wide ligand recognition spectrum as mammalian class A SR (Pearson et al., 1995). *In vitro* studies demonstrated its importance in bacteria binding and phagocytosis of both Gram-positive and Gram-negative bacteria, but not yeast (Pearson et al., 1995; Rämet et al., 2001). Moreover, after bacterial challenge, transcription of *dSr-CI* is upregulated, further supporting its role in host defence (Irving et al., 2005). Interestingly, the *dSr-CI* locus presents a high polymorphism, likely a consequence of positive selection (Lazzaro, 2005). This feature could be the consequence of an arms race as often observed for host-pathogen interactions. However, the role of dSr-CI in bacterial phagocytosis has never been properly confirmed with the use of null mutations.

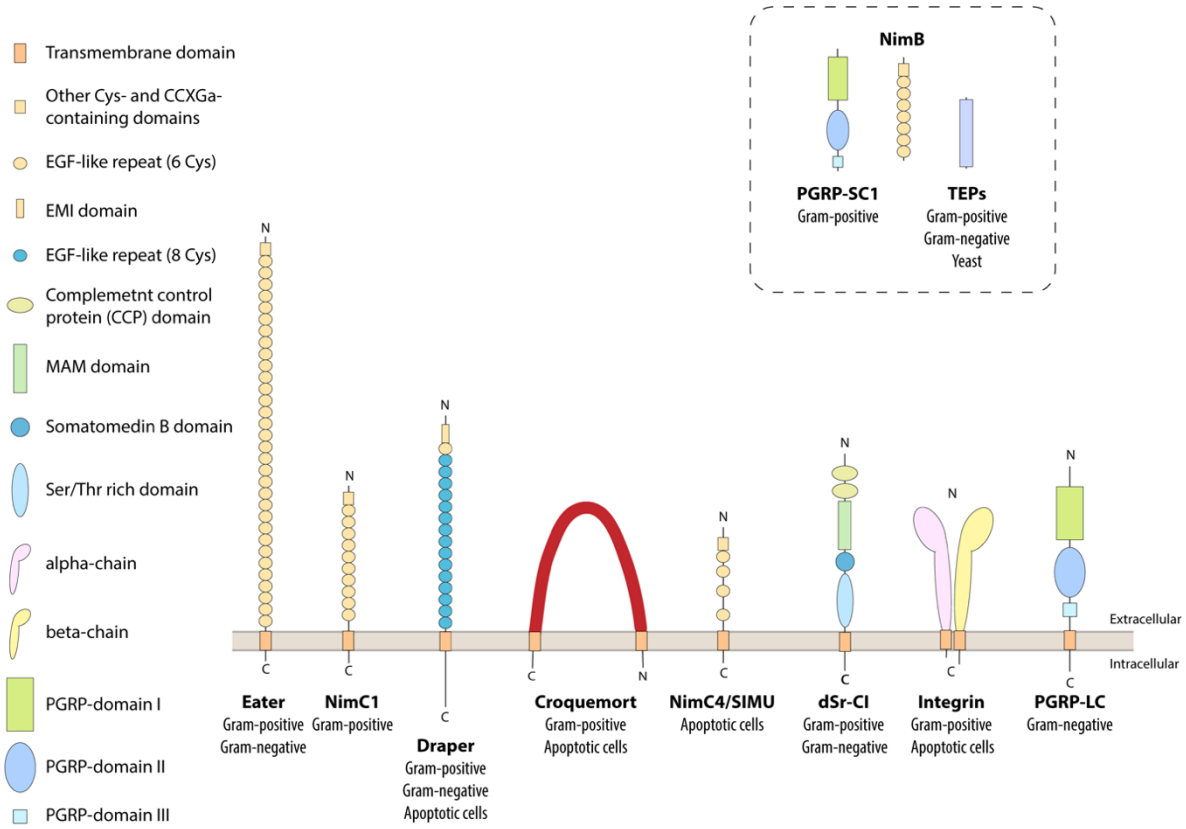

Figure 3. Plasma-membrane phagocytic receptors and opsonins in *Drosophila*. Graphical illustration of the main Drosophila phagocytic receptors involved in microbe engulfment and efferocytosis (see main text for further details). The dashed box represents potential secreted opsonins mediating phagocytosis in *Drosophila.*

The *Drosophila* genome encodes twelve class B SRs with homology to the mammalian CD36, which was initially discovered in apoptotic cell clearance (Savill et al., 1992). Many of the class B SRs are expressed in the fly gut, but their function is poorly characterized (Herboso et al., 2011). One CD36 member, Croquemort (Crq), is specifically expressed in plasmatocytes and has been shown to function as a receptor for apoptotic cells (Franc et al., 1999, 1996). However, the molecular mechanisms underlying its action and its cognate ligand on apoptotic cells are still unknown. Further *in vitro* work in *Drosophila* S2 macrophage-like cells has revealed the involvement of Crq in *Staphylococcus aureus* recognition and uptake (Stuart et al., 2005). Adult *crq* knockout flies show protracted and increased *Diptericin* and *Unpaired3* expression during microbial infections and under unchallenged conditions, suggesting that Crq might indirectly alter the humoral response (Guillou et al., 2016). Nevertheless, the role of Crq as an engulfing receptor has been recently challenged by several subsequent studies, which revealed a contribution of Crq in phagosome maturation rather than in particle recognition (Guillou et al., 2016; Han et al., 2014; Meehan et al., 2016). Finally, RNAi-mediated silencing of *crq* in plasmatocytes was shown to block lipid uptake, suggesting that Crq may be involved

in the acquisition of lipids, as shown for other CD36 members (Woodcock et al., 2015). Considering that lipid metabolism plays a key role in various aspects of phagocytosis (e.g. phagosome formation and maturation) (Yeung et al., 2006), it cannot be ruled out that Crq involvement in lipid scavenging might indirectly affect the phagocytic process. Several RNAi based screens on S2 *Drosophila* cells have identified another class B SR, Peste, as specifically required for the engulfment of *Mycobacterium fortuitum* and *Listeria monocytogenes*, but not *Escherichia coli* or *S. aureus* (Agaisse et al., 2005; Philips et al., 2005). The lack of *Peste* null mutants did not allow to validate these RNAi studies and fully test the relevance of this receptor in host defence.

1.3.2.2 Peptidoglycan recognition proteins (PGRPs)

PGRPs form a family of pattern recognition receptors involved in the detection of the bacterial cell wall component peptidoglycan (PGN). Some PGRPs possess enzymatic activities (amidase), cleaving peptidoglycans into non-immunogenic fragments (Mellroth et al., 2003). Other non-catalytic PGRPs can bind peptidoglycan but cannot cleave it as they lack a critical cysteine in the catalytic pocket. In insects, these non-catalytic PGRPs function as pattern recognition receptors dedicated to the identification of bacteria upstream of Toll and Imd pathways, two NF-kB pathways involved in the regulation of antimicrobial peptide genes (Royet et al., 2011). Early RNAi screens in S2 cells identified PGRP-LC as a key molecule involved in the uptake of Gram-negative, but not Gram-positive bacteria (Rämet et al., 2002). PGRP-LC is a pattern recognition receptor that recognizes DAP-type peptidoglycan found in the wall of Gram-negative and certain Gram-positive (*Bacillus*) bacteria (Stenbak et al., 2004). In contrast to the initial results obtained by RNAi, following studies using RNAi approaches (Kocks et al., 2005) failed to confirm a major role of PGRP-LC in the phagocytosis of Gram-negative bacteria.

1.3.2.3 Nimrods

An interesting and relatively newly discovered family of phagocytic receptors in *Drosophila* comprises proteins characterized by the presence of the so-called Nimrod (NIM) repeats (**Fig. 4**). The NIMs are a subtype of the epidermal growth factor (EGF) repeat, which often function in adhesion, coagulation, and receptor-target interactions (Bork et al., 1996). The *Nimrod* gene family comprises twelve members, ten of which cluster on the second chromosome, and two, *eater* and *draper*, on the third chromosome. According to their domain structure, proteins belonging to this family can be classified into different categories. The *draper*-type genes

(NimA and *draper*) encode proteins that carry only one NIM domain, followed by several EGF repeats, and one Emilin (EMI) domain at their N-terminal end (Callebaut et al., 2003). The Draper protein was initially identified in a genetic screen as being regulated by Glial Cells Missing (Gcm), a key glial transcription factor (Freeman et al., 2003). It is expressed on the surface of *Drosophila* plasmatocytes and several non-professional phagocytes, such as glia and epithelial cells. Draper possesses 15 extracellular EGF repeats, a single transmembrane domain, and a signalling cytosolic tail. Draper has been reported to recognize apoptotic cells by interacting with either PS or the endosomal protein Pretaporter, that are found on the surface of dying cells (Kuraishi et al., 2009; Tung et al., 2013). During development, Draper mediates not only the removal of dying neurons, but it is also involved in neuronal pruning and synapse clearance (Awasaki et al., 2006; Kurant et al., 2008; Logan et al., 2012; Manaka et al., 2004; Ziegenfuss et al., 2008). Moreover, it plays a key role in nurse cell clearance by epithelial follicle cells in the *Drosophila* ovary (Serizier and McCall, 2017). The role of Draper in apoptotic cell clearance is evolutionarily conserved. Homologues of Draper are CED-1 in *C. elegans* (Mangahas & Zhou, 2005), MEGF10 in humans and JEDI in mouse (Hamon et al., 2006). Subsequent studies implicated Draper in the phagocytosis of both *S. aureus* and *E. coli* bacteria (Cuttell et al., 2008; Hashimoto et al., 2009; Shiratsuchi et al., 2012). In particular, Hashimoto et al. identified bacterial lipoteichoic acid as a ligand for Draper in *S. aureus* engulfment by plasmatocytes (Hashimoto et al., 2009).

Another Nimrod sub-family, the Nimrod C-type (Nimrod C1-4, Eater) proteins, are transmembrane receptors containing multiple NIM domains and lacking the classical EGF repeats. Previous studies have implicated some Nimrod C-type proteins in the phagocytosis of apoptotic cells (NimC4, also called Six-Microns-Under (SIMU)) (Kurant et al., 2008) and bacteria (Eater and NimC1) (Kocks et al., 2005; Kurucz et al., 2007a).

SIMU (NimC4) is highly expressed on embryonic macrophages, glia, and ectoderm. SIMU acts upstream of Draper, triggering the engulfment of apoptotic cells through PS recognition (Kurant et al., 2008; Shklyar et al., 2013). However, the detailed molecular mechanisms of interaction between SIMU and Draper remain poorly understood, since no direct interaction between the two proteins has been proven. This would suggest that other factors might be required for the SIMU/Draper-mediated embryonic efferocytosis.

So far, Eater and NimC1 represent the best characterized Nimrod-C type receptors for bacterial uptake. Eater has initially been identified by Kocks and colleagues in a genetic screen as a target of the transcription factor Serpent, which is necessary for bacterial phagocytosis (Kocks et al., 2005). Eater possesses 32 NIM repeats in its extracellular region and has a small cytosolic

tail with unknown function (Kocks et al., 2005), although predicted phosphorylation sites are present. This receptor is specifically expressed on both larval and adult *Drosophila* plasmatocytes, as well as in S2 cells. Several studies using RNAi or an overlapping set of deficiencies removing *eater* and 7 flanking genes, have pointed to its crucial role in the phagocytosis of Gram-negative and Gram-positive bacteria (Horn et al., 2014; Kocks et al., 2005; Nehme et al., 2011), as well as the elimination of bacteria entering the hemolymph by crossing the gut (Nehme et al., 2011). By using a soluble Fc-tagged receptor variant, Chung et al. reported that Eater directly binds to Gram-positive bacteria, and also to Gram-negative bacteria after Cecropin A pre-treatment. Nevertheless, the specific bacterial molecules recognized by Eater remain unknown (Chung and Kocks, 2011). Recently, an *eater* deficient mutant generated by homologous recombination confirmed the importance of Eater in the phagocytosis of Gram-positive, but not Gram-negative bacteria (Bretscher et al., 2015). Moreover, *eater* null plasmatocytes are not sessile and less adhesive (Bretscher et al., 2015). Nevertheless, how the Eater-mediated adhesion properties relate to its phagocytic ability is unknown.NimC1 was initially identified as a target of P1, a plasmatocyte-specific antibody (Kurucz et al., 2007a). NimC1 has 10 EGF-like repeats, a single transmembrane domain, and a cytosolic region with predicted phosphorylation sites. Initial RNAi studies on NimC1 provided evidence for its involvement in the phagocytosis of *S. aureus* bacteria (Kurucz et al., 2007a). Moreover, immunofluorescence-based flow cytometry assays showed that NimC1 binds to bacteria *in vitro* (Zsámboki et al., 2013).

1.3.2.4 Integrins

Integrins are heterodimeric proteins composed by α and β subunits, and are involved in a wide range of cellular processes, such as cell spreading and motility. In addition to their adhesion tasks, integrins were shown to mediate phagocytosis of different types of particles in several organisms. The *Drosophila* genome encodes five α- and two β-integrin subunits (Brown et al., 2000). In 2011, Nagaosa and colleagues showed the implication of βv integrin in the phagocytosis of both apoptotic cells and *S. aureus* in *Drosophila* (Nagaosa et al., 2011). Moreover, by using cell wall deficient bacteria, the authors revealed peptidoglycan as the surface determinant recognized by integrins during phagocytosis. Genetic and biochemical studies have subsequently revealed that the αPS3 and βv integrin subunits function as a

heterodimer in the phagocytosis of both apoptotic cells and *S. aureus* bacteria (Nonaka et al., 2013).

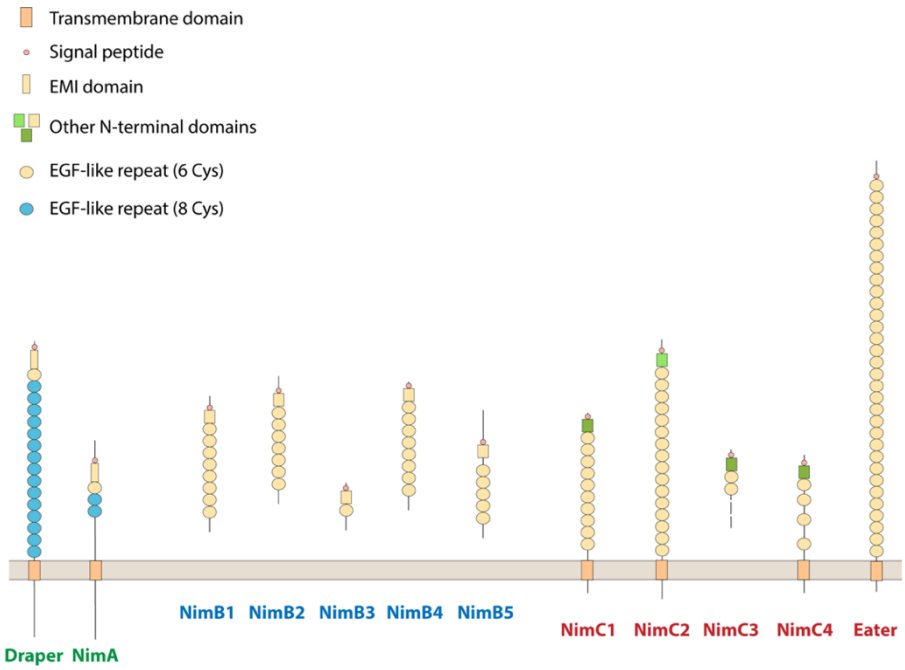

Figure 4. The *Drosophila* Nimrod family. Nimrod is a family of PRRs characterized by the repetition in their protein sequence of a specific EGF-like repeat, called NIM. Moreover, according to other structural features, members of this family can further be classified into NimA (Draper and NimA), NimB (NimB1-5), and NimC (NimC1-4, Eater) types. NimA, NimB1-5 and NimC1-4 are clustered on the second chromosome, whereas Draper and Eater are localized on the third chromosome.

1.3.2.5 Opsonins in Drosophila

Opsonins are molecules that bind to microbes and favour their engulfment by macrophages. In mammals, complement factors C3b or antibodies efficiently opsonize microbes promoting their uptake by macrophages through dedicated receptors. Little is known on the role of opsonins in microbial phagocytosis in *Drosophila*. However, the *Drosophila* genome encodes six thioester-containing proteins (Teps) structurally related to the mammalian complement factor C3 family. *Tep* genes are specifically expressed in plasmatocytes, fat body, and some barrier epithelia (Bou Aoun et al., 2011). Four of them, Tep1-Tep4, contain a signal peptide, indicating that they are secreted proteins and could function as opsonins. *Tep5* is thought to be a pseudogene (Bou Aoun et al., 2011), while *Tep6* (also called *Mcr*) plays a role in gut septate junctions (Batz et al., 2014) and is therefore unlikely to play any role in phagocytosis, although one study suggested otherwise (Stroschein-Stevenson et al., 2006). Three members of this family (Tep1, Tep2 and Tep4) show a strong upregulation after bacterial challenge, because they are regulated by the stress-responsive JAK-STAT pathway (Lagueux et al., 2000). The

importance of the Tep proteins in insect phagocytosis has been first demonstrated in the mosquito *A. gambiae* (Blandin et al., 2004). A further RNAi study in *Drosophila* S2 cells (Stroschein-Stevenson et al., 2006) shed light on the function of some Tep members in binding and enhancing phagocytosis of *E. coli* (Tep2), *S. aureus* (Tep3) and *Candida albicans* (Tep6). Additional evidence in support of opsonisation in insects comes from work using a compound mutant lacking the four immune-inducible Teps (i.e. Tep1-4) (Dostálová et al., 2017). The authors found that Teps operate in both the humoral and cellular response of *Drosophila* immunity, by promoting Toll pathway activation and phagocytosis of Gram-positive bacteria (Dostálová et al., 2017). Therefore, it is likely that some Teps can bind to microbes and promote phagocytosis, as recently reported for Tep4 in *P. aeruginosa* infection (Haller et al., 2018). Despite these recent results, the specific receptors mediating the uptake of Teps remain unknown.

Another class of potential secreted opsonins are the Nimrod B type proteins, whose function remains to date poorly characterized. This subclass comprises five Nimrod B members (NimB1-B5) that contain multiple NIM domains. Close relatives of the *Drosophila NimB* genes with immune functions are found in other insect species (Estévez-Lao and Hillyer, 2014; Ju et al., 2006; Matsunaga and Fujiwara, 2002; Midega et al., 2013). Moreover, *Drosophila* NimB1 and NimB2 proteins have been shown to bind bacteria *in vitro* (Zsámboki et al., 2013). A recent study has shown that NimB5 is produced by the fat body to regulate plasmatocyte adhesion and proliferation rate. NimB5 is induced upon starvation and adjusts plasmatocyte numbers to the metabolic state of the host. Phagocytosis is however normal in *NimB5* deficient larvae (Ramond et al., submitted). Finally, evidence from the literature points to a potential role of PGRP-SC1A as a secreted opsonin for *S. aureus* bacteria and Toll pathway activation (Garver et al., 2006). Use of null mutations in the *PGRP-SC1* gene cluster did not reveal any role in Toll activation (Paredes et al., 2011). As PGRP-SC degrades peptidoglycan, it might nevertheless affect bacterial cell wall structure and consequently phagocytosis.

1.3.2.6 Down Syndrome Cell Adhesion Molecule 1 (DSCAM1)

Dscam1 is part of the Immunoglobulin (Ig) family, and possesses 95 variable exons, grouped into clusters. These clusters are in turn flanked by constant exons, leading to about 36000 potential isoforms due to alternative splicing (Armitage et al., 2012; Möller et al., 2013). Dscam1 has initially been implicated in the development of the nervous system and neuron wiring, where isoform-isoform specific interactions shape the dendritic pattern. (Wojtowicz et al., 2004; Zhan et al., 2004). In 2000, Schmucker and colleagues proved that infection leads to

the production of specific secreted isoforms, with different potential "recognition abilities" that can be detected in the *Drosophila* hemolymph (Schmucker et al., 2000). However, a recent study did not reveal any change of *Dscam1* splicing upon infection (Armitage et al., 2014). Use of an RNAi targeting all *Dscam* isoforms in whole flies or plasmatocytes showed that Dscam1 is involved in binding and uptake of *E. coli* by hemocytes, and suggested that this protein might act as an opsonin (Watson et al., 2005). Moreover, *Dscam* isoforms have been implicated in the immune response of the mosquito *Anopheles gambiae* (Dong et al., 2006). Although conceptually appealing, the implication of Dscam in the fly immune response awaits further confirmation.

1.3.3 Uptake machinery and phagosome formation

Particle engulfment through phagocytosis implies an active and dynamic remodelling of the plasma membrane, which is mainly guided by the actin cytoskeleton (**Fig. 5**). Past studies on mammalian phagocytosis have shown that the engulfment of a receptor-bound particle can happen via different movements of the plasma membrane: i) "zippering", typical for IgG-opsonized Fcγ-mediated phagocytosis, ii) "sinking" C3-mediated phagocytosis, or iii) "triggered" bacterial internalization by macropinocytosis (see (Flannagan et al., 2012) for a detailed review). Particle internalization by Fcγ receptor (FcγR) is the most well characterized mechanism of engulfment yet. In 2003, Pearson and colleagues proved morphological similarities between *Drosophila* plasmatocyte- and mammalian macrophage-mediated phagocytosis (Pearson et al., 2003). They observed that engulfment of *S. aureus* bacteria was primarily mediated by zippering of the plasma membrane around the microbe, suggesting that bacterial uptake occurs mainly via receptor clustering and activation. However, macropinocytosis-like and sinking (i.e. without pseudopod formation) uptake events of bacteria were also observed. Those data indicate that similar mechanisms to the mammalian C3-mediated phagocytosis exist in the fruit fly, and that *Drosophila* plasmatocytes might be able to induce bacterial engulfment without formation of a phagocytic cup. In the same study, by performing a genetic screen for hemocyte phagocytosis mutants, the authors identified various proteins required for efficient bacterial engulfment, previously known to be involved in mammalian cytoskeletal reorganization. They demonstrated that the nucleation-promoting factors D-SCAR and D-WASP are important in the engulfment of *S. aureus*. Those proteins are the *Drosophila* homologues of WAVE and WASP, which activate the Arp2/3 complex responsible for F-actin generation at the engulfment site. Other regulators of the actin network

dynamics have been identified to mediate bacterial phagocytosis in *Drosophila*, such as the Rho GTPases Cdc42, Rac1, and Rac2 (which activate the nucleation factors D-SCAR and D-WASP), the Arp2/3 complex and Profilin (Agaisse et al., 2005; Avet-Rochex et al., 2005; Pearson et al., 2003; Philips et al., 2005; Stroschein-Stevenson et al., 2006; Stuart et al., 2005). Besides factors regulating membrane rearrangement, the coat-protein complex I and II (COPI, COPII) (Rämet et al., 2002; Stuart et al., 2007), as well as the exocyst complex (Stuart et al., 2007), have been shown to act as potential regulators during *Drosophila* phagocytosis.

Actin polymerization and plasma membrane remodelling around the targeted particle are tightly regulated by specific signalling pathways, which are triggered after the engagement of the engulfing receptor. Draper signalling following bacteria or apoptotic cell binding is one of the most well characterized intracellular cascades in *Drosophila* (Cuttell et al., 2008; Etchegaray et al., 2012; Hashimoto et al., 2009; Ziegenfuss et al., 2008). When glial cells phagocytose apoptotic cells, Draper induces Src42-mediated phosphorylation of the ITAM motifs located in the intracellular domain of the receptor. Next, Shark (the *Drosophila* homologue of the Syk kinase) binds via its SH2 domain to Draper's phosphorylated sites, thereby activating other downstream signals needed for apoptotic cell engulfment (Ziegenfuss et al., 2008). Draper-mediated phagocytosis of apoptotic cells is also coupled with Ca^{2+} release from the endoplasmic reticulum via the Ryanodine receptor Rya-r44F (Cuttell et al., 2008). This event leads to extracellular Ca^{2+} influx, which is regulated by the *Drosophila* junctophilin Undertaker, Ca^{2+} channels, and the Ca^{2+} sensor dSTIM (Cuttell et al., 2008). Interestingly, a recent study showed that Draper transcriptional levels in glia are induced by a signalling pathway downstream of Toll-6 upon binding with its ligand Spz5. As Spz5 is produced by dying neurons, this suggest a mechanism by which apoptotic cells prime glial cells to promote their efferocytosis (McLaughlin et al., 2019). Upon *S. aureus* uptake, Draper signals via Rac1 or Rac2, which are required for bacterial engulfment. Nevertheless, this study did not uncover any downstream role of Shark during Draper-mediated Gram-positive bacterial phagocytosis (Hashimoto et al., 2009).

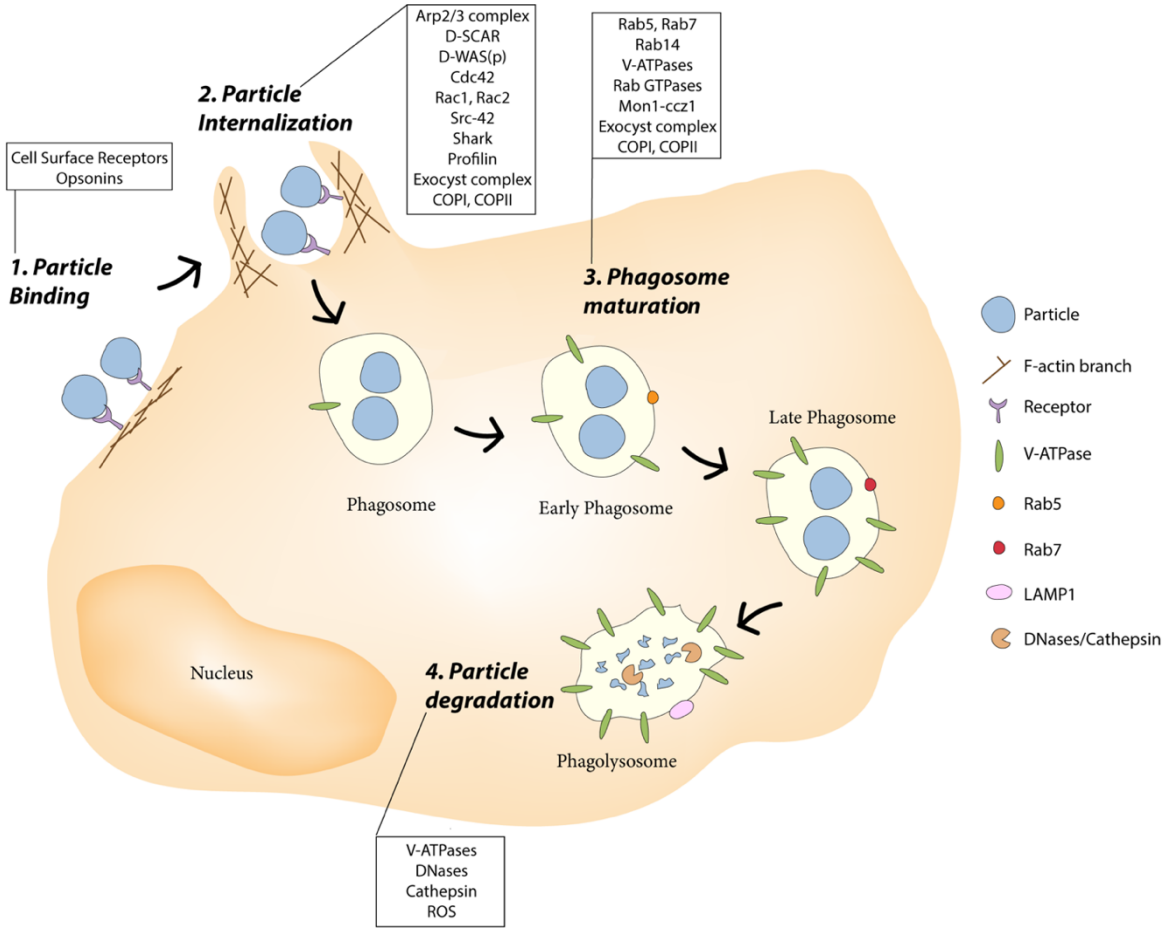

Figure 5. The phagocytosis process in *Drosophila*. (1-2) Recognition of the target particle by surface receptors on *Drosophila* professional phagocytes leads to F-actin branching at the engulfment site, resulting in the formation of the so-called phagocytic cup. Actin polymerization progresses around the particle, until the phagocytic protrusions fuse at the leading edges, generating a newly formed phagosome. This initial plasma membrane-derived vacuole does not have the ability to digest the internalized material. (3) Newly formed phagosomes, indeed, undergo a series of subsequent fission and fusion events (called phagosome maturation) with cellular organelles (early endosomes, late endosomes and lysosomes) (3). Rab5 is a key regulator of the initial fusion events. Another GTPase, Rab7, is needed for the late phagosome-lysosome fusion. Phagosome maturation culminates in the formation of a highly acidic phagolysosome (4). During this last step, the phagolysosome acquires important components for the final particle destruction step, such as DNAses and proteases. Boxes for each step show the main factors that have been involved in *Drosophila* phagocytosis.

1.3.4 Phagosome maturation and microbe destruction

Newly formed phagosomes undergo a maturation process in order to acquire bactericidal activity. The phagosome matures through fusion with endosomes and lysosomes (**Fig. 5**). These organelles release their hydrolase content into the phagosome lumen, a process involving small GTPases of the Rab family (Kinchen and Ravichandran, 2010; Li et al., 2009; Nieto et al., 2010). Interestingly, a comparative proteomics analysis revealed that a high percentage of phagosome-associated proteins, including Rabs, are conserved between mammals and *Drosophila* S2 cells, making these cells a suitable model for phagosome maturation studies

(Stuart et al., 2007). Rab5 is generally considered a regulator of the initial fusion events, by tethering early endosomes to the newly formed phagosome (Alvarez-Dominguez et al., 1996; Duclos et al., 2000; Jahraus et al., 1998; Kinchen et al., 2008; Kitano et al., 2008; Vieira et al., 2001). Both *in vitro* and *in vivo* works on *Drosophila* confirmed the role of Rab5 in the initial steps of bacterial phagocytosis and phagosome maturation (Agaisse et al., 2005; Cheng et al., 2005; Horn et al., 2014; Peltan et al., 2012; Philips et al., 2005). As the phagosome matures, Rab5 is replaced by Rab7 in a process called Rab conversion. Different studies in worms, yeast and *Drosophila* have shown that Rab conversion might be regulated by the Rab5 effector complex Sand-1/Mon1-Ccz1 (Kinchen and Ravichandran, 2010; Poteryaev et al., 2010, 2007; Wang et al., 2002; Yousefian et al., 2013). However, a study in *Drosophila* plasmatocytes revealed that Rab7 recruitment to the phagosome might be regulated by Rab14. This GTPase localizes to both early Rab5- and late Rab7-positive *S. aureus* phagosomes, and mutant flies for *rab14* show impaired phagosome maturation (Garg and Wu, 2014). Once recruited, Rab7 is responsible for inducing phagolysosome formation. This final part of the process involves a complex of proteins named HOPS (Homotypic Fusion and Protein Sorting), which is composed of the six subunits: Vps11, Vps16, Vps18, Vps33, Vps39 and Vps41 (Akbar et al., 2011; Kinchen et al., 2008; Nickerson et al., 2009). Mutations affecting the *full-of-bacteria* (*fob*) gene, encoding the Vps16 *Drosophila* homologue, cause defects in phagosome-lysosome fusion and increase susceptibility to bacterial infection (Akbar et al., 2011; Vieira et al., 2003). A characteristic protein present on the phagolysosome membrane in mammals is Lysosomal-associated membrane protein 1 (LAMP1), which is needed for lysosome fusion with the phagosome (Garin et al., 2001; Huynh et al., 2007; Peltan et al., 2012). A *Drosophila* homolog of Lamp1 exists but has not been tested for its involvement in phagocytosis.

A key feature of phagosome maturation consists of its progressive lumen acidification, which is allowed by the activity of proton pumping vacuolar ATPase (V-ATPase). V-ATPases are found on the phagosome from the initial stages of its formation and their activity allows reaching a final pH of around 4.5-5. This final phagosome acidification is essential for proper activation of lysosomal hydrolases and consequent microbe destruction. In *Drosophila*, several V-ATPase subunits have been involved in phagosome acidification, and therefore bacterial clearance inside the phagocytes (Cheng et al., 2005; Philips et al., 2005). Proteases, notably cathepsins, are other important factors acquired through phagosome-lysosome fusion, and required for particle degradation. Cathepsin activity has been detected in *Drosophila* S2 cell phagosomes, suggesting that these cysteine proteases might be active in lysosomal compartments and participate in the fly's microbe destruction (Kocks et al., 2003). In addition,

DNase II, an enzyme involved in DNA degradation, was shown to participate in bacterial clearance in phagolysosomes of adult flies (Seong et al., 2006). By using fluorescent probes, Myers et al. revealed a hemocyte reactive oxygen species response following bacterial infection. In particular, the authors could distinguish between an early ROS response, which was more pronounced in non-phagocytic cells, and a late and protracted response specific for hemocytes that have engulfed bacteria (Myers et al., 2018).

Finally, a putative glutamate transporter named Polyphemus (Polyph) is required in plasmatocytes to successfully control bacterial growth during an infection. *polyph* mutants show altered intracellular transport of glutamate in plasmatocytes, leading to higher internal ROS, decreased phagocytosis ability, and resistance to pathogenic infection (Gonzalez et al., 2013).

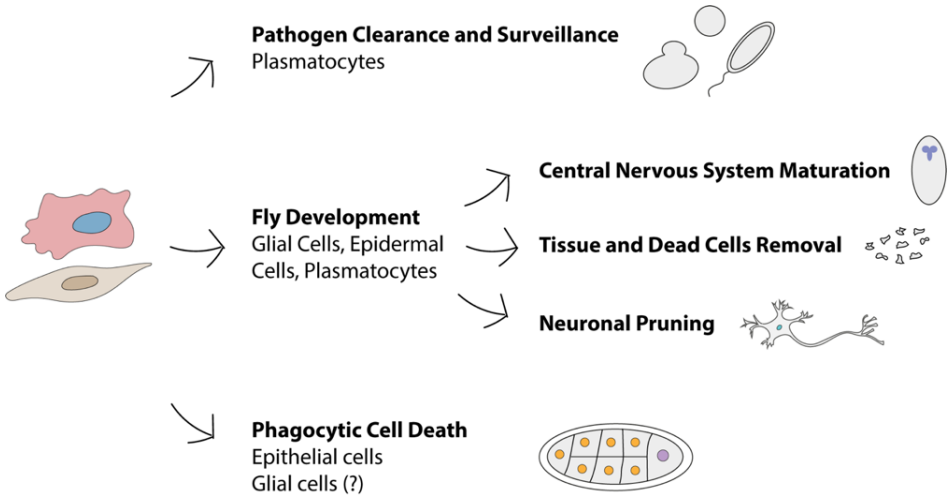

Figure 6. Physiological roles of phagocytosis in *Drosophila*. Professional (red cell) and non-professional (brown cell) phagocytes contribute to fly homeostasis and survival against infections. Bacterial clearance is mainly performed by the macrophage-like plasmatocytes (red). However, *Drosophila* possesses several types of non-professional phagocytes (brown) that regulate diverse aspects of fly development. During development, phagocytosis represents a key determinant for remodelling and shaping several organs. Starting at embryogenesis, mainly glia plays a critical role in CNS maturation by removing apoptotic neurons and pruned neuronal branches. Finally, recent evidence supports the existence of phagocytic cell death in the *Drosophila* ovary, where the epithelial follicle cells surrounding the egg chamber induce nurse cell death.

1.3.5 Physiological roles of phagocytosis in *Drosophila*

Phagocytosis by *Drosophila* professional and non-professional phagocytes has critical impacts on the animal's survival, development, and homeostasis (**Fig. 6**). In the following paragraphs, we will describe the physiological relevance of phagocytosis during i) macrophage-mediated bacterial destruction (Shandala et al., 2013), ii) elimination of apoptotic neurons by glia

(Etchegaray et al., 2016), iii) neuronal pruning by epidermal cells (Han et al., 2014), and iv) germline cell clearance by epithelial cells in the ovary (Meehan et al., 2016).

1.3.5.1 Phagocytosis of bacteria and fungi during the cellular immune response

Drosophila lives in a microbe-rich environment, and therefore needs powerful defence mechanisms to fight invading pathogens. Phagocytosis constitutes a major cellular branch of the innate immune response, which is mediated by the plasmatocytes (**Figs. 6-7**). Studies in mutant larvae and in phagocyte-depleted flies have demonstrated the importance of *Drosophila* phagocytosis in the survival against certain microbes including yeast, Gram-negative and -positive bacteria (Braun, Hoffmann, & Meister, 1998; Charroux & Royet, 2009; Defaye et al., 2009; Elrod-Erickson S.; Schneider, D., 2000). In 2007, Brennan and colleagues identified a mutation called *psidin* (*phagocytes signalling impaired*) that affects a plasmatocyte lysosomal protein required to destroy internalized bacteria. Interestingly, this protein was also required for the fat body-mediated expression of Defensin upon septic injury, demonstrating that plasmatocytes contribute to Defensin expression in the fat body during both Gram-positive and Gram-negative infections (Brennan et al., 2007). Other studies found that plasmatocytes are also required for the activation of a systemic antimicrobial response in the fat body upon oral but not systemic infection (Basset et al., 2000; Charroux and Royet, 2009). It has also been proposed that plasmatocytes clear the hemolymph from the entry of intestinal microbiota or pathogens. Indeed, plasmatocyte deficient flies have higher numbers of bacteria in the hemolymph and are more susceptible to oral infection with *Serratia marcescens* (Braun et al., 1998; Nehme et al., 2007).

Plasmatocytes might rely on different recognition and signalling processes in order to efficiently clear the infection. It has been suggested that two receptors, Draper and an integrin, mediate the phagocytosis of the Gram-positive bacterium *S. aureus* in a dual recognition mechanism, by respectively recognizing lipoteichoic acid and peptidoglycan (Shiratsuchi et al., 2012).

Past studies in S2 cells allowed to identify important genes involved in the *Drosophila* cellular immune response against fungal pathogens (Levitin et al., 2007; Stroschein-Stevenson et al., 2006). An RNAi approach in S2 cells has identified specific genes required for successful recognition and uptake of *C. albicans* (Stroschein-Stevenson et al., 2006). Microarray studies allowed to identified a translational regulator, d4E-BP, as a key player in host defence against *C. albicans* (Levitin et al., 2007). d4E-BP is encoded by the *Thor* gene, which was found to be one of the most up-regulated genes in S2 cells co-incubated with *C. albicans* (Levitin et al.,

2007). *Drosophila d4E-BP*^{null} flies showed increase sensitivity for *C. albicans*, but not *Saccharomyces cerevisiae* infection (Levitin et al., 2007). Moreover, the importance of phagocytosis in fly defence against fungal pathogens, has been observed upon Zygomycetes infection (Halder et al., 2008).

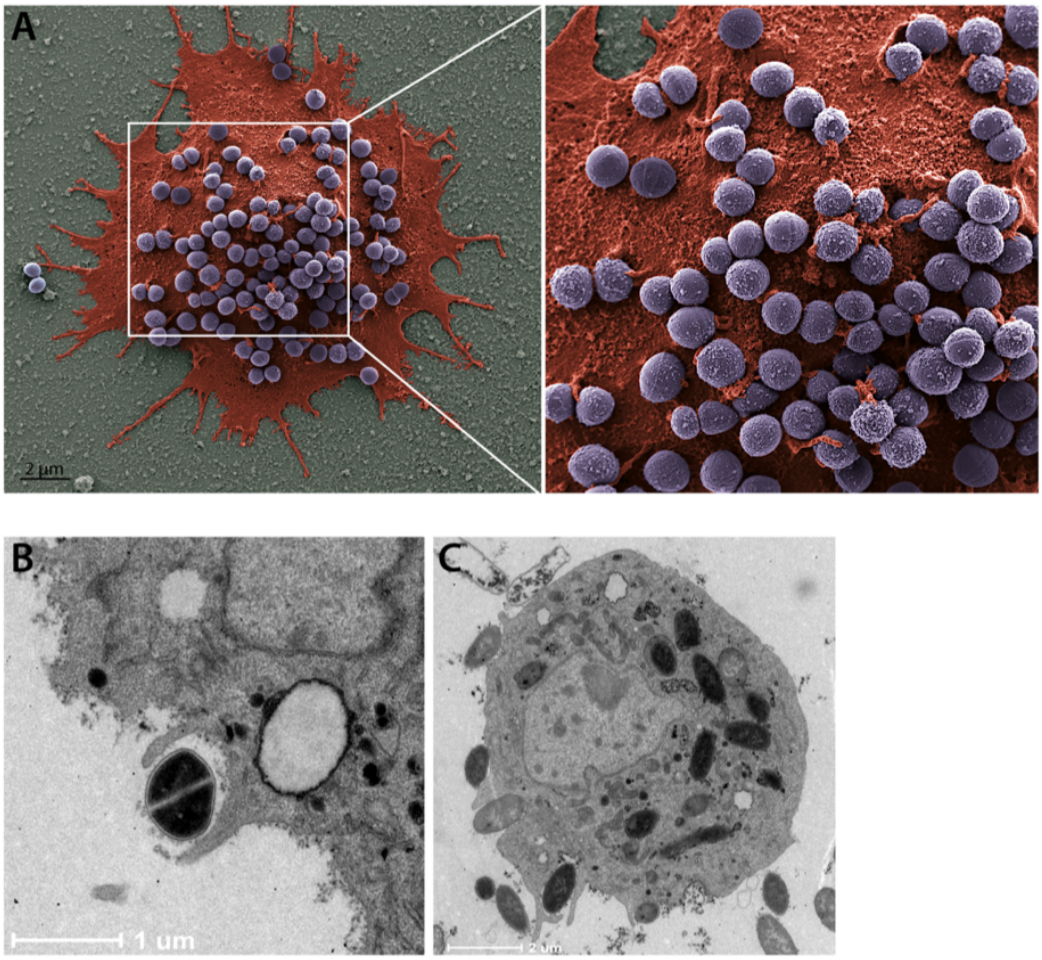

Figure 7. Plasmatocyte-mediated bacterial phagocytosis. (A) Plasmatocytes are *Drosophila* professional phagocytes, sharing functional features with mammalian macrophages. This scanning electron micrograph shows a plasmatocyte (red) from a third instar w^{1118} *Drosophila* larva engulfing *S. aureus* bacteria (purple). (B-C) Transmission electron micrographs of w^{1118} third instar larvae engulfing *S. aureus* (B) and *E. coli* (C) bacteria. The electron micrographs were taken in collaboration with the BioEM facility at the École Polytechnique Fédérale de Lausanne.

Finally, it is noteworthy to mention that even though plasmatocytes have been described as the first line of defence in bacterial clearance, several pathogens have evolved strategies to escape destruction by phagocytosis. In this scenario, the phagocyte rather becomes a host cell for bacteria to proliferate in and establish the infection. *Drosophila* plasmatocytes can harbour several intracellular bacteria, in particular *M. marinum* and *L. monocytogenes* (Dionne et al., 2003; Mansfield et al., 2003). Interestingly, phagocyte-mediated sequestration of *Salmonella typhimurium* bacteria (also called "phagocytic encaspulation") was shown to act as a powerful

resilience mechanism (Shinzawa et al., 2009). Phagocytic encapsulation of intracellular pathogens, which is dependent on the *Drosophila* p38 MAP kinase (Dmp38b), limits the exposure of host tissues to bacteria, and thus severe pathogenic damage. As a consequence, Dmp38b-mediated tolerance extends host survival without targeting the bacterium (Shinzawa et al., 2009).

1.3.5.2 Efferocytosis during Drosophila development

Clearance of apoptotic cells by phagocytosis is an important component of *Drosophila* development that ensures the remodelling and shaping of different organs, such as the CNS or imaginal discs. Moreover, phagocytosis of apoptotic cells by embryonic plasmatocytes is required for their maturation with regards to the tissue damage response and bacterial infection (Weavers et al., 2016a).

Defects in the clearance of apoptotic cells have pathological consequences. For example, *draper* mutant flies accumulate undegraded apoptotic neurons inside glial cells throughout the lifespan, which leads to age-dependent neurodegeneration (Etchegaray et al., 2016). Therefore, as a cell undergoes apoptosis, a very tightly regulated molecular signalling process is activated, in order to rapidly remove the dying cell in a silent manner by the immune system. This pathway consists on multiple steps (**Fig. 8**), which appear to be evolutionarily conserved.

In the following paragraphs, I will describe the well-characterized maturation of the CNS via glial phagocytosis, by first giving a general overview on the distinct stages of phagocyte-mediated apoptotic cell recognition.

Apoptotic cell recognition

Upon programmed cell death induction, the dying cell releases “find-me” signals which are involved in the attraction of phagocytes to the site of apoptosis. Those secreted molecules were first discovered in mammals mainly through *in vitro* studies, and most of them have been shown to be caspase-dependent. Examples of molecules that are able to enhance phagocytes localization to the dying cells are the lipid lysophosphatidylcholine (Lauber et al., 2003), sphingosine 1-phosphate (Gude et al., 2008), the fractalkine CX3CL1 (Truman et al., 2008) and the nucleotide ATP and UDP (Elliott et al., 2009). Whether all those molecules act in a concerted or redundant way is still object of investigations. Despite the importance of those signals have been extensively demonstrated in mammals, no evidences so far report the presence of similar pathways in *Drosophila*. Nevertheless, hemocyte migration properties in response to chemotactic factors have been previously investigated, especially during

embryogenesis (Wood and Jacinto, 2007). In addition to migratory movements during development, embryonic hemocytes are rapidly recruited to epithelial wounds, in a process that resembles vertebrate inflammation. The nature of the chemoattractant responsible of the hemocytes recruitment to wounds has been extensively discussed and its nature is still largely debated (Moreira et al., 2010; Weavers et al., 2016b).

Once the macrophages are brought in proximity to the site of apoptosis through find-me signals, they are able to specifically recognize the dying cell through the so called eat-me signals. Apoptotic cells are indeed characterized by an alteration of their surface or by the exposure of specific molecules that allow phagocytes to discriminate between a healthy and a dying cell. Those molecules are recognized by engulfment receptors on the macrophage, which bind to them either directly or indirectly through bridging molecules. After binding, the intracellular engulfment machinery is activated and leads to the actin-dependent engulfment of the dying cell. One of the best characterized and evolutionarily conserved eat-me signal is phosphatidylserine (PS), reported to be present in human, worms and *Drosophila* (Fadok et al., 2001, 1998; Shklyar et al., 2013; Tung et al., 2013; Wu et al., 2006). PS is a phospholipid which is normally present in the cytoplasmic leaflet of healthy cells plasma membrane through aminophospholipid translocase activity. However, in the early stages of apoptosis, PS is exposed on the outer leaflet of the cell membrane, as a consequence of aminophospholipid translocase inhibition. Even though apoptotic cells engulfment mediated by eat-me signal recognition is a tight and efficient mechanism, it can happen that healthy cells transiently expose eat-me signals on their surface. For this reason, normal living cells are provided of inhibitory mechanisms, or “don’t eat-me” signals, to prevent the engulfment of living cells from phagocytes. Examples of don’t eat-me signals in mammals are CD31, 46 and 47 (Brown et al., 2002; Gardai et al., 2005; Poon et al., 2014). To date, no evidence for don’t eat-me molecules have been reported in *Drosophila*, neither in nematodes. However, a study on the *Drosophila* embryonic CNS development suggest a mechanism by which living cells transiently exposing eat-me signals on their membrane are not removed. The authors showed that the presence of PS alone on the surface of a cell was not enough to trigger its own engulfment by phagocytes, and that caspase activity is also required for efficient engulfment (Shklyar et al., 2013). A recent study demonstrated the importance of dying cell clearance in the embryo by showing that excessive levels of apoptotic cells can lead to defects in hemocyte migration along the ventral line, as well as defective wound responses (Roddie et al., 2019).

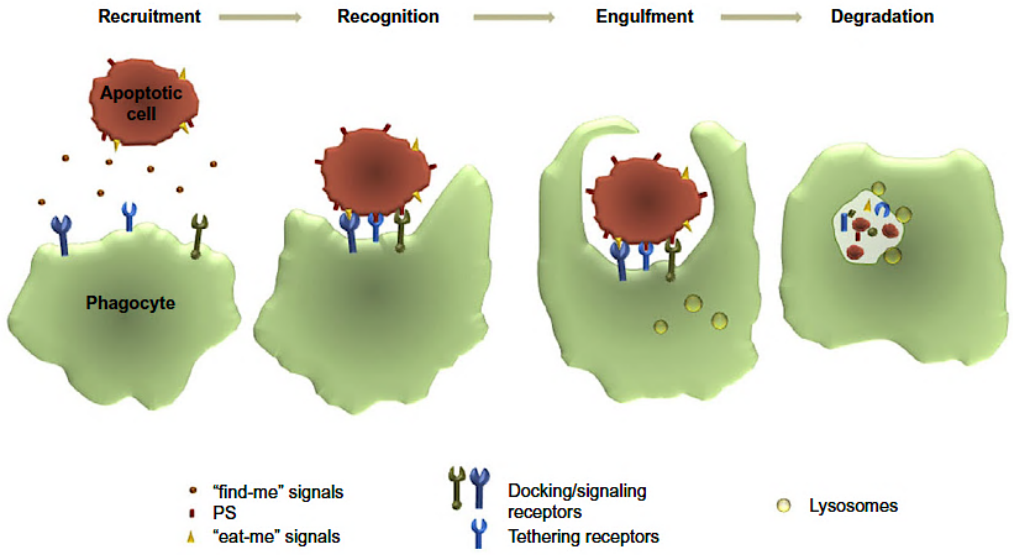

Figure 8. Schematic representation of different stages of apoptotic cell clearance. Upon programmed cell death induction, find-me signals are secreted by the dying cells, which also exposes eat-me signals. The interaction between eat-me signals and dedicated receptors on the phagocyte membrane leads to receptor clustering, which is followed by apoptotic cell engulfment and degradation in the phagosome (from Shklover et al., 2015a).

Glial phagocytosis during *Drosophila* nervous system maturation

The development of complex nervous systems is characterized by the generation of surplus neurons which are eliminated through apoptosis and subsequent phagocytic clearance. In parallel to the adjustment of neuronal cell number, shaping of the CNS in both vertebrates (O'Leary and Koester, 1993) and invertebrates (Tissot and Stocker, 2000) involves the removal of excessive neuronal branches, a process called pruning. In the *Drosophila* CNS, neuronal cell number is adjusted throughout the fly development at three main stages: embryogenesis, metamorphosis and emerging adult (Abrams et al., 1993; Rogulja-Ortmann et al., 2007; Tissot and Stocker, 2000; Togane et al., 2012). Glia of ectodermal origin are considered the main phagocytes responsible for shaping the CNS (Kurant et al., 2008), similar to their vertebrate counterparts, microglia and astrocytes (**Fig. 9**). During the late stages of *Drosophila* embryogenesis, around 30% of neurons undergo apoptosis (Abrams et al., 1993; Rogulja-Ortmann et al., 2007) and are efficiently removed by glial cells (Kurant et al., 2008). The phagocytic ability of embryonic glia to engulf and degrade apoptotic neurons is determined by the phagocytic receptors SIMU and Draper, whose genes are regulated by two transcription factors Gcm and Repo (Shklyar et al., 2014). Different studies performed in *simu* and *draper* mutant embryos have demonstrated that SIMU is required for recognition and engulfment of apoptotic neurons by glia, whereas Draper is mostly needed for their degradation (Freeman et al., 2003; Kurant et al., 2008; Shklyar et al., 2014). It has been shown that the *Drosophila* JNK pathway in glial cells can enhance clearance of apoptotic neurons, by promoting their

degradation without interfering with the expression of SIMU and Draper (Shklover et al., 2015b). During metamorphosis, CNS development is characterized by two different types of neuronal efferocytosis: phagocytosis of apoptotic neurons and neuronal pruning. Removal of apoptotic neurons in pupae takes place during the first quarter of metamorphosis, and it is also mediated by glial cells (Hilu-Dadia et al., 2018; Tasdemir-Yilmaz and Freeman, 2014). Professional phagocytes seem not to be involved in efferocytosis in the CNS at this developmental stage (Cantera and Technau, 1996; Hilu-Dadia et al., 2018; Tasdemir-Yilmaz and Freeman, 2014). Interestingly, recent work has shown that Draper is required for both engulfment and degradation of dying neurons by glia (Hilu-Dadia et al., 2018; Tasdemir-Yilmaz and Freeman, 2014). On the contrary, SIMU, although highly expressed at this developmental stage, is not present in glia but rather on macrophages outside the CNS. The authors suggest that Draper might activate two distinct signalling pathways to trigger removal of apoptotic neurons. One is the JNK pathway in astrocytes and in ensheathing glia, while the other is still unknown (Hilu-Dadia et al., 2018). Pruning of larval axons during metamorphosis is mediated by the glial receptor Draper and Ced-6, and is not caspase dependent (Awasaki et al., 2006). Interestingly, the more recent studies demonstrate that astrocytes use distinct molecular programs to engulf neuronal debris originated from neuronal death or local pruning (Hilu-Dadia et al., 2018; Tasdemir-Yilmaz and Freeman, 2014). The last phase of neuronal elimination takes place in emerging flies (Peterson et al., 2002). However, the cells responsible for neuron removal are still unknown. A mechanism proposed by Kato and collaborators suggested that dying neurons are able to induce proliferation of the surrounding glia, which eventually participate in their engulfment (Kato et al., 2009). Flies deficient for the two main receptors for apoptotic cells, *draper* and *simu*, are however viable but their lifespan is reduced and their CNS shows accumulation of apoptotic bodies.

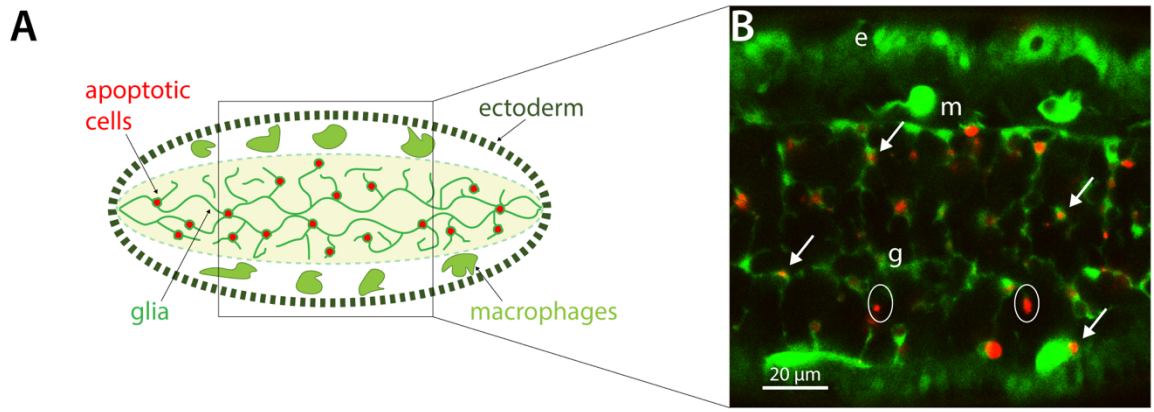

Figure 9. Phagocytic cell populations in the late *Drosophila* embryo. (A) Schematic ventral view of stage 16 embryo, showing that at this developmental stage apoptosis is largely restricted to the CNS (thin dashed oval). Large dashed oval depicts ectodermal cells, red dots represent AnnexinV-labeled apoptotic cells (inside or outside green-labelled phagocytes), glial cells are depicted as green network inside CNS, macrophages are shown as big green cells outside CNS, showing that they do not enter the CNS at this stage. (B) Confocal snapshot of stage 16 live embryo at the area depicted as black frame in A. Ectoderm (e), glia (g) and macrophages (m) are labelled with a simu-cytGFP transgenic construct. Apoptotic cells are labelled by injected fluorescent AnnexinV (red). Most of AnnexinV-positive cells are found inside phagocytic glia and macrophages (arrows). Circles highlight unengulfed AnnexinV-labelled cells. Image source: courtesy from E. Kurant.

Peripheral dendrite clearance by epidermal cells

In *Drosophila*, peripheral neurons undergo dendrite and axon pruning as part of developmental remodelling during metamorphosis. In this context, the degenerating dendrites are efficiently removed by epidermal cells, rather than by professional phagocytes or glia, even though plasmatocytes are found in contact with those neurons (Han et al., 2014). Recognition of the degenerating dendrites by epidermal cells is mediated by the engulfing receptor Draper. Interestingly, loss of function of *crq* in epidermal cells does not affect dendrite uptake, but rather phagosome maturation. The authors also identified an additional CD36 family protein, Debris Buster, required for late stages of phagosome maturation (Han et al., 2014).

Phagocytosis mediated-cell death in *Drosophila*, an insight from the fly ovary

Since its discovery, phagocytosis has always been thought to be a beneficial mechanism to remove unwanted cells or infecting microbes. However, recent advances in the phagocytosis field have shed light on specific mechanisms whereby living cells can be taken up by phagocytes. One of those processes has been named “phagoptosis”, or “primary phagocytosis” (Brown and Neher, 2012). Phagoptosis has been defined as phagocyte mediated cell death, where phagocytes induce death of living cells by mediating their engulfment. It has been suggested that phagoptosis is used among several multicellular organisms to mediate both

homeostatic functions and cellular stress responses (Brown and Neher, 2012). However, if not tightly regulated, phagoptosis can lead to pathological states. A well characterized mechanism of mammalian phagoptosis is given by microglial engulfment of viable neurons, leading to their "killing" and consequent neurodegenerative conditions. In *Drosophila*, phagocytic receptor expression in glia must be tightly regulated, as expression of SIMU in adult glia (where it is not normally expressed) or overexpression of Draper triggers neuronal degeneration by phagoptosis of living cells (E. Kurant, personal communication). This study reveals that dysregulation of phagocytic receptors in glia can promote neurodegeneration by inducing neuronal loss.

Recent studies on the *Drosophila* ovary have given a first hint of the presence of phagoptosis in that organ (Etchegaray et al., 2012; Timmons et al., 2016). The *Drosophila* ovary represents a well-established system for studying efferocytosis mediated by non-professional neighbouring cells, since no circulating macrophages are present in that organ. The egg chamber is the single unit of the ovary, and it consists of three main cell types: nurse cells, oocyte and follicle cells. While nurse cells and oocyte are germline-derived, follicle cells are somatic epithelial cells that form a protective barrier around the egg chamber. Late oogenesis in *Drosophila* is characterized by a developmental form of non-apoptotic death of the nurse cells. In this physiological context, Timmons and colleagues showed that the phagocytic machinery of the follicle cells is required to induce the death of the nurse cells, as well as their subsequent removal (Timmons et al., 2016). As a further proof of this mechanism, inhibition of the phagocytosis genes in follicle cells impaired germline death and clearance. These findings indicate that phagoptosis might occur in *Drosophila* as a physiological mechanism of developmental cell death.

1.4 Objectives of the present PhD thesis

The aim of this doctoral thesis was to provide a deeper characterization of two Nimrod receptors, Eater and NimC1, in the *Drosophila* cellular immune response. In particular, this work aimed to further elucidate i) the involvement of each receptor in bacterial phagocytosis and ii) the role of Eater in hemocytes sessility and adhesion.

Since the initial study of Kurucz and colleagues (Kurucz et al., 2007a), NimC1 involvement in phagocytosis has always been investigated by the use of RNAi and overlapping deficiencies. Here, we revisited its function by generating a loss-of-function mutation in

NimC1 by homologous recombination. Moreover, we generated a double mutant *NimC1*^{1}*,eater*^{1} to better elucidate the role and individual contribution of each receptor to phagocytosis. The results from this first part of my thesis resulted in a publication in *The FEBS Journal* (Melcarne et al., 2019).

The second objective of my thesis addresses the potential signalling function of Eater in hemocyte sessility. This Nimrod receptor has been reported to play a key role in hemocytes adhesion to the larval integument (Bretscher et al., 2015). However, its role in sessility has not been characterized during adulthood yet. Moreover, it is still unknown whether Eater fulfils its function by working as a cell adhesion molecule or as a signalling receptor, activating specific downstream pathways for cell spreading. Therefore, in this part of the thesis we further characterized Eater requirement for hemocyte sessility in adult blood cells by using the previously described *eater*^{1} mutant (Bretscher et al., 2015), and by analysing the effects of Eater over-expression in hemocyte spreading by generating novel transgenic animals containing the *eater* gene downstream of the *UAS* promoter. In addition, we performed transcriptomics analysis on *eater*^{1} larval hemocytes to elucidate Eater's role in signalling hemocytes adhesion. The results from this second part are presented as an unpublished manuscript.

Chapter 2: Two Nimrod receptors, NimC1 and Eater, synergistically contribute to bacterial phagocytosis in *Drosophila melanogaster*

Two Nimrod receptors, NimC1 and Eater, synergistically contribute to bacterial phagocytosis in *Drosophila melanogaster*

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Running title: Bacterial phagocytosis in *Nimrod* receptors mutants

Abbreviations: Nim, Nimrod; Hml, Hemolectin; EdU, 5-ethynyl-2′-deoxyuridine; SEM, scanning electron microscopy; TEM, transmission electron microscopy; PTU, phenylthiourea; DAPI, 4',6- diamidino-2-phenylindole; RT-PCR, reverse transcription polymerase chain reaction

Key words: *Drosophila*, phagocytosis, hemocytes, Nimrod, adhesion, innate immunity

Abstract

Eater and NimC1 are transmembrane receptors of the *Drosophila* Nimrod family, specifically expressed in hemocytes, the insect blood cells. Previous *ex vivo* and *in vivo* RNAi studies have pointed to their role in the phagocytosis of bacteria. Here, we have created a novel *NimC1* null mutant to re-evaluate the role of NimC1, alone or in combination with Eater, in the cellular immune response. We show that NimC1 functions as an adhesion molecule *ex vivo*, but in contrast to Eater it is not required for hemocyte sessility *in vivo*. *Ex vivo* phagocytosis assays and electron microscopy experiments confirmed that Eater is the main phagocytic receptor for Gram-positive, but not Gram-negative bacteria, and contributes to microbe tethering to hemocytes. Surprisingly, *NimC1* deletion did not impair phagocytosis of bacteria, nor their adhesion to the hemocytes. However, phagocytosis of both types of bacteria was almost abolished in $NimC1^{1};eater^{1}$ hemocytes. This indicates that both receptors contribute synergistically to the phagocytosis of bacteria, but that Eater can bypass the requirement for NimC1. Finally, we uncovered that NimC1, but not Eater, is essential for uptake of latex beads and zymosan particles. We conclude that Eater and NimC1 are the two main receptors for phagocytosis of bacteria in *Drosophila,* and that each receptor likely plays distinct roles in microbial uptake.

Introduction

Phagocytosis is an ancient and evolutionarily conserved process, generally defined as the cellular uptake of particles bigger than 0.5 μm. Phagocytosis is an important feeding mechanism in primitive and unicellular organisms, such as amoeba [1]. In higher organisms, phagocytosis is performed by dedicated cells (phagocytes) and is used as a powerful process to internalize and eliminate pathogens, as well as to trigger host inflammation [2]. Moreover, phagocytosis contributes to tissue homeostasis and embryonic development, mainly via the removal of apoptotic corpses [3]. Phagocytosis is a complex membrane-driven process guided by the actin cytoskeleton of the host phagocytic cell. It involves the recognition and subsequent binding of the microbe by surface receptors. These interactions are essential to activate intracellular signalling pathways that finally culminate in the formation of the phagosome [4]. Several studies have highlighted similarities between the phagocytic machinery of *Drosophila* and mammals, such as the involvement of actin and actin-related proteins [5–7]. *D. melanogaster* harbours highly efficient phagocytes, called plasmatocytes, which originate from multipotent progenitors (prohemocytes). In healthy larvae, prohemocytes can differentiate into two mature hemocyte types: plasmatocytes and crystal cells. While the later are involved in the

melanization response [8], plasmatocytes are professional phagocytes sharing functional features with mammalian macrophages, and represent the most abundant hemocyte class at all developmental stages. They play a key role in bacterial clearance during infection, as well as in the removal of apoptotic corpses [9,10]. The ability of *Drosophila* hemocytes to perform efficient phagocytosis, relies on the expression of specific cell surface receptors that can bind particles and induce their engulfment. While many receptors have been implicated in bacterial phagocytosis, their specific involvement or individual contribution is less clear [11,12]. In this paper, we have characterized the phagocytic role of NimC1 and Eater, two EGF-like repeat Nimrod surface receptors specifically expressed in hemocytes [13,14]. The Nimrod family of proteins is characterized by the presence of epidermal growth factor (EGF)-like domains, also called "NIM repeats". This family comprises a cluster of ten genes (NimA, NimB1-5 and NimC1-4) encoded by genes clustered on the chromosome II, and two related hemocyte surface receptors, Eater and Draper, encoded by genes on chromosome 3 [14,15]. Early studies have shown the implication of some Nimrod C-type proteins in bacterial phagocytosis (Eater and NimC1) [13,14,16] or engulfment of apoptotic bodies (Draper and NimC4/SIMU) [17,18]. More recently, the Eater transmembrane receptor has also been involved in hemocyte adhesion and sessility [16]. Nimrod C1 (NimC1) is a 90 kDa transmembrane protein characterized by 10 NIM repeats in its extracellular region, a single transmembrane domain and a short cytosolic tail with unknown function [14]. NimC1 has been initially identified as the antigen of a hemocyte-specific antibody (P1), being involved in phagocytosis of bacteria [14]. Kurucz and colleagues showed that *NimC1* silencing by RNAi decreases *Staphylococcus aureus* uptake by plasmatocytes, whereas its overexpression in S2 cells enhances phagocytosis of both *S. aureus* and *Escherichia coli* bacteria and makes the cells highly adherent [14]. Here, we generated a null mutation in *NimC1* by homologous recombination (called $NimC1^{1}$) and re-visited its function in hemocyte-mediated immunity. Moreover, we recombined the *NimC1* mutation with the previously described $eater^{1}$ mutant [16], generating a $NimC1^{1};eater^{1}$ double mutant. Using these genetic tools, we first show the involvement of NimC1 in *ex vivo* cell adhesion and in the regulation of hemocytes proliferation. Contrasting with previous RNAi studies [14], our *ex vivo* phagocytosis assays demonstrate that NimC1 is not required for phagocytosis of Gram-positive or Gram-negative bacteria. Nevertheless, we show that this Nimrod receptor contributes to the uptake of latex beads and zymosan yeast particles. The use of the $NimC1^{1};eater^{1}$ double mutant not only re-confirmed Eater as the main Gram-positive engulfing receptor, but, more importantly, revealed a synergistic action of NimC1 and Eater in microbe phagocytosis. $NimC1^{1};eater^{1}$ hemocytes from third instar larvae, failed indeed to phagocytose

any type of bacteria. Collectively, our study points to a major role of NimC1 and Eater in the phagocytosis of bacteria, and suggests that those proteins likely play distinct roles in microbial uptake, as tethering and docking receptors.

Results

Generation of a *NimC1* null mutant by homologous recombination

In order to characterize *NimC1* functions, we generated a null mutant by deleting the corresponding *NimC1* gene region. The deletion removes the ATG translation start site and the following 852 bp sequence. The knockout was performed in the w^{1118} background, using homologous recombination [19], which also lead to the insertion of a 7.9 kb cassette carrying the $white^{+}$ gene (Fig. 10 A,B). Functional deletion of *NimC1* was confirmed by RT-PCR performed on total RNA and by P1 (anti-NimC1 antibody [14]) immunostaining (Fig. 10 C,D). As *NimC*1 is specifically expressed in hemocytes and has been implicated in phagocytosis, we combined the *NimC1* mutation with the previously described $eater^{1}$ null mutant [16], generating a double mutant $NimC1^{1};eater^{1}$ (Fig. 10). Both $NimC1^{1}$ and $NimC1^{1};eater^{1}$ flies were viable and did not show any developmental defect. For over-expression studies, we also generated flies containing the *NimC1* gene downstream of the *UAS* promoter. Using these tools, we characterized the function of NimC1 focussing on hemocytes of third instar larvae.

NimC1 deficient hemocytes show adhesion defects *in vitro*

Eater has been involved in hemocyte adhesion and sessility [16]. Given the structural similarities between NimC1 and Eater [14], we first investigated the role of NimC1 in cell adhesion. We observed that the cell area of $NimC1^{1}$ and $eater^{1}$ adherent hemocytes was decreased compared to that of w^{1118} wild-type control (Fig. 11A) [16]. Notably, the cell area of $NimC1^{1};eater^{1}$ adherent hemocytes was significantly smaller than that of single mutants. Quantification analysis revealed that wild-type hemocytes have a mean cell area of 237 μm^2, while $NimC1^{1}$, $eater^{1}$ and $NimC1^{1};eater^{1}$ mutants have 120, 114 and 99.7 μm^2, respectively (Fig. 11B). Image-based cytometry analysis of free-floating hemocytes revealed that the spreading defects observed in our mutants were not due to an inherently smaller cell size (Fig. 11C). In order to get a deeper insight into these adhesion defects, we investigated hemocytes morphology by scanning electron microscopy (SEM). Lamellipodia are a key feature of highly motile cells, playing a central role in cell movement and migration [20]. They represent flat cellular protrusion, characterized by an enriched network of branched actin filaments.

Filopodia, instead, are rather used by the cell to sense the surrounding microenvironment, and consist of parallel actin filaments that emerge from the lamellipodium. Spread plasmatocytes from wild-type larvae appeared as round adherent cells with a central bulge within the cell body, from which lamellipodia and filopodia extended (Fig. 11D). *NimC1* and *eater* null hemocytes were still able to form narrow filopodia projections. However, both single and double mutants showed an obvious lamellipodium decreased region compared to wild-type control (Fig. 11D). Collectively, our results point to a role of NimC1 in hemocyte spreading and lamellipodia extension.

In the *Drosophila* larva, circulating hemocytes can attach to the inner layer of the cuticle, forming striped patterns along the dorsal vessel, and lateral patches in association with the endings of peripheral neurons [8,21–23]. These sub-epidermal sessile compartments are known as hematopoietic pockets [21,23–26]. Previous work has shown that *eater* larvae lack the sessile hemocyte compartment and have all peripheral hemocytes in circulation [16]. To further investigate whether the *NimC1* deletion affects sessility, we explored hemocyte localization using the hemocyte marker *HmlΔGal4>UAS-GFP* by whole larva imaging and cross section visualization. In *NimC1^{1},HmlΔGal4,UAS-GFP* third instar (L3) wandering larvae, hemocytes were still able to enter the sessile state, forming dorsal and lateral patches (Fig. 11E,F). In contrast, both *eater^{1}* and *NimC1^{1};eater^{1}* larvae lacked sessile hemocytes, all plasmatocytes being in circulation (Fig. 11E,F). *In vivo* RNAi targeting *NimC1* confirmed the hemocyte adhesion defect observed with the null mutant (Fig. 11G,H). This indicates that the observed phenotypes were indeed caused by the deletion of *NimC1* and not the genetic background. Altogether, our data indicate that NimC1 contributes to hemocyte adhesion *ex vivo*, but in contrast to Eater, it is not directly required for hemocyte sessility *in vivo*.

NimC1 null larvae have an increased number of hemocytes

Drosophila hematopoiesis occurs in two successive waves. A first set of hemocytes is produced during embryogenesis, giving rise to a defined number of plasmatocytes and crystal cells. This embryonic hemocyte population expands in number during the following larval stages. The second hemocyte lineage derives from the lymph gland, a specialized organ that develops along all larval stages. The lymph gland acts as a reservoir of both prohemocytes and mature hemocytes, which are released at the onset of metamorphosis or upon parasitization [8,27–30]. Finally, accumulating evidence suggests that also the sessile hematopoietic pockets function as an active peripheral hematopoietic niche [21,23,26]. In order to further investigate the role of NimC1, and its potential interaction with Eater in peripheral hematopoiesis, we counted by

flow cytometry the number of the all peripheral hemocytes populations (i.e. both sessile and circulating). Larvae containing the hemocyte marker *Hml*Δ*dsred.nls*, combined with the *NimC1* and *eater* null mutants, were used (Fig. 12A-C). Our study confirmed that *eater* L3 wandering mutant larvae have more hemocytes than the wild type [16] (Fig. 12A). Similarly, *NimC1*^{1} third instar larvae have 3.2 times more circulating hemocytes compared to the wild-type (Fig. 12A). As *NimC1*^{1} L2 larvae have a wild-type like number of hemocytes, the increase in hemocyte counts in this mutant takes place at the end of larval development (Fig. 12C). Surprisingly, hemocyte number was 6 times higher in *NimC1*^{1}*;eater*^{1} double mutant L3 larvae (Fig. 12A), suggesting that *eater* and *NimC1* additively regulate hemocyte counts. A higher hemocyte number was already observed in second instar larvae in the double mutant (Fig. 12C). We next investigated whether also lymph glands from third instar mutant larvae had an increased number of mature hemocytes compared to wild-type. Visual count of *Hml*Δ*dsred.nls* positive cells from fixed primary lymph gland lobes revealed no major differences between mutants and wild-type, although a decreased trend in hemocyte number in single and double mutants could be observed (Fig. 12D,E). In agreement with this observation, primary lymph gland lobes of *eater* and *NimC1* mutants showed a modest reduced area compared to control, which was not statistically significant (Fig. 12F). Nevertheless, the ratio of *Hml*Δ*dsred.nls* positive cells to the all primary lymph gland cell population (i.e. DAPI positive), was not significantly altered between wild-type and mutants. (Fig. 12G).

We then decided to explore whether the increase in peripheral hemocytes count observed in our mutants (Fig. 12A-C) was caused by a higher proliferation rate. EdU incorporation experiments revealed that *NimC1*^{1} and *eater*^{1} single mutants have a higher frequency of peripheral proliferating hemocytes compared to wild-type in middle L3 but not L2 larvae (Fig. 12H,I). The higher proliferation rates might therefore explain the increased number in hemocyte counts in both L3 wandering (Fig. 12A) and middle L3 (Fig. 12B) larvae. Interestingly, we found that both hemocyte count and mitotic rate were higher in *NimC1*^{1}*;eater*^{1} in L2 and L3 larvae indicating that both receptors additively regulate hemocyte proliferation levels (Fig. 12A-C, H, I). The higher hemocyte count in *NimC1* mutant larvae was phenocopied when using an *in vivo* RNAi approach to silence *NimC1* (Fig. 12J). Of note, over-expression of *NimC1*, using the *Hml*Δ*-Gal4* plasmatocyte driver, did not increase the peripheral hemocyte count (Fig. 13A), nor their adhesion properties (Fig. 13B-D). Over-expression of *NimC1* in hemocytes from *eater* deficient larvae did not rescue the lack of sessility phenotype and the *ex vivo* adhesion defect caused by the absence of *eater* (Fig. 13E).

We also investigated a possible role of NimC1 in crystal cell and lamellocyte differentiation.

Crystal cells are the second hemocyte type present in non-infected larvae, specifically involved in the melanization response and wound healing [31]. Crystal cells can be found in both the sessile and circulating state. Recent studies have shown that a fraction of those cells derive from sessile plasmatocyte by transdifferentiation [26]. Consequently, crystal cells need sessile plasmatocytes to be themselves sessile [16]. Lamellocytes are barely present in healthy larvae, but can differentiate from plasmatocytes [25,32] or prohemocytes [33] in response to specific stress signals, such as parasitization. They are thought to play an essential role in encapsulation of parasitoid wasp eggs. Our study indicates that *NimC1* mutants retain the ability to differentiate fully mature crystal cells (Fig. 14). Moreover, our data also show that the *NimC1* deletion does not affect the ability to encapsulate parasitoid wasp eggs (Fig. 15). Finally, we did not uncover any role of NimC1 in the systemic antimicrobial response of larvae against Gram-positive (*Micrococcus luteus*) or Gram-negative bacteria (*Erwinia carotovora carotovora*), as revealed by the wild-type like induction of *Diptericin* and *Drosomycin* gene expression, two target genes of the Imd and Toll pathways, respectively [34] (Fig. 16).

NimC1 contributes with Eater to phagocytosis of bacteria

A previous *in vivo* RNAi approach had revealed a role of NimC1 in the phagocytosis of Gram-positive bacteria [14]. We used the *NimC1* deletion to further elucidate the requirement of this receptor in bacterial uptake by performing *ex vivo* phagocytosis assays at two different time points (early-30 minutes and late-60 minutes). As previously reported [16], *eater* null mutant hemocytes were impaired in their capacity to phagocytose the Gram-positive bacterium *S. aureus* (Fig. 17A,B), but not the Gram-negative bacterium *E. coli* (Fig. 17C,D). In contrast to the previous RNAi experiments [14], loss of *NimC1* affected neither the phagocytosis of *S. aureus* nor that of *E. coli* (Fig. 17A-D). However, hemocytes derived from *NimC1;eater* mutant larvae were not only severely impaired in the phagocytosis of *S. aureus* (Fig. 17A,B), as expected, but also of *E. coli* (Fig. 17C,D). This indicates that Eater and NimC1 contribute redundantly to the phagocytosis of Gram-negative bacteria, as the presence of Eater or NimC1 is able to compensate for the absence of the other. The use of a double mutant also revealed a contribution of NimC1 to the phagocytosis of *S. aureus*, although Eater plays the predominant role. To further confirm these phagocytosis defects, we extended the analysis to two additional Gram-positive (*Staphylococcus epidermidis*, *M. luteus*) (Fig. 17E,F) and one Gram-negative (*Serratia marcescens*) (Fig. 17G) bacteria. Phagocytosis of all those microbes was not impaired in $NimC1^{1}$ null hemocytes. However, $NimC1^{1};eater^{1}$ double mutant hemocytes showed a strongly reduced phagocytosis for both the Gram positive bacteria *S. epidermidis* and *M. luteus*

(Fig. 17E,F), and the Gram-negative bacterium *S. marcescens* (although statistically non-significant due to high variability of the wild-type) (Fig. 17G). Those data further confirmed our initial findings (Fig. 17A-D). Interestingly, *NimC1* null hemocytes showed a higher phagocytic index, when compared to wild-type, for *S. epidermidis* and *M. luteus* bacteria. We hypothesized that the absence of NimC1 could trigger a compensatory pathway in plasmatocytes, specific for certain bacteria, in order to fulfil NimC1 phagocytic functions. The signalling of this putative compensatory pathway, that would eventually finally lead to a higher bacteria uptake by plasmatocytes, might be dependent on Eater, given the dramatically reduced phagocytic ability of the double mutant.

Eater and NimC1 receptors play a critical role in adhesion to bacteria

To better understand the cause of $eater^{1}$ and $NimC1^{1},eater^{1}$ phagocytosis defects, and thereby to elucidate the unique role of these receptors in bacterial uptake, we performed scanning and transmission (TEM) electron microscopy experiments. Both these techniques allow following the different membrane-driven events during the phagocytosis process. Hemocytes from the corresponding genotypes were incubated with either *E. coli* or *S. aureus* live bacteria for 30 minutes to evaluate bacterial adhesion by SEM, and to follow bacterial uptake at 60 minutes by TEM. In wild-type and $NimC1^{1}$ hemocytes incubated with *S. aureus*, we observed plasma membrane remodelling, with the formation of a phagocytic cup and pseudopod protrusions that progressively surrounded bacteria, finally leading to their engulfment (Fig. 18A,B white arrowheads). Similar observations were made for *wild-type*, $NimC1^{1}$, and $eater^{1}$ hemocytes incubated with *E. coli* bacteria (Fig. 18C,D). Surprisingly, upon incubation of $eater^{1}$ and $NimC1^{1};eater^{1}$ hemocytes with *S. aureus*, no bacteria were present on the cell surface (Fig. 18A). A decreased level of bacteria adherence was also observed in $NimC1^{1};eater^{1}$ hemocytes incubated with *E. coli* (Fig. 18C). In accordance with SEM experiments, transmitted electron micrographs showed numerous engulfment events in *wild-type* and $NimC1^{1}$ hemocytes with *S. aureus* bacteria (Fig. 18B, arrows), as well as for *E. coli* in *wild-type*, $NimC1^{1}$, and $eater^{1}$ hemocytes (Fig. 18D). Altogether, these experiments point to the importance of Eater in binding Gram-positive bacteria, which is consistent with a previous report [35], but also to a redundant role of NimC1 and Eater in binding Gram-negative bacteria. Furthermore, they suggest that these two receptors do not play any critical role in bacteria internalization, as *NimC1;eater* mutant showed (rare) engulfment events (Fig. 18B,D arrows).

To further confirm the bacteria adhesion defects, we incubated hemocytes and live fluorescent bacteria either on ice or with Cytochalasin D. Both treatments inhibit the engulfment process,

without altering the binding of the bacteria to the phagocytic cell [6]. In both conditions (Fig. 18E,F), we observed less bacteria binding to plasmatocytes in the same genotypes that were defective for phagocytosis in our *ex vivo* assays (*eater*^{1} for *S.* aureus, and *NimC1*^{1}*;eater*^{1} for *S. aureus* and *E. coli*, Fig. 17A-D).

Phagocytosis of Latex Beads and Zymosan yeast particles is impaired in *NimC1* null mutants

To further understand the role of Eater and NimC1 in the phagocytosis process, we proceeded to analyse the uptake of "neutral" latex beads particles. We also tested their role in the phagocytosis of zymosan, a compound found on the cell wall of yeast. While bacteria present at their surface specific targets for the engulfing receptors, latex beads can be seen as non-immunogenic particles, that do not bear any ligands for the phagocyte. We observed that the phagocytic index of latex beads was wild type-like in *eater* null plasmatocytes. Interestingly, plasmatocytes lacking the NimC1 receptor showed a significantly reduced ability to engulf latex beads, as well as zymosan yeast particles (Fig. 19A,B). Thus, we could uncover a phagocytic defect in the *NimC*1 single mutant only when using particles that do not display bacterial motifs, suggesting that bacteria can bypass NimC1, probably by recruiting other phagocytic receptors, such as Eater.

Bacteria adhesion and latex beads engulfment are not impaired in *croquemort* and *draper* mutant hemocytes

The drastic effect observed with the *NimC1*^{1}*;eater*^{1} double mutant on phagocytosis and bacteria adhesion led us to explore the contribution of other previously characterized phagocytic receptors using the same assays. Draper and Croquemort are two transmembrane receptors expressed by plasmatocytes, and belong to the Nimrod and CD36 family, respectively [14,36]. With SIMU (NimC4), they both play a key role in the engulfment of apoptotic bodies [18,36–39]. Moreover, a role in *S. aureus* phagocytosis has also been described for Draper and Croquemort, as well as in *E. coli* phagocytosis for Draper [17,40–42]. Although we did observed a modest decrease in *S. aureus* phagocytosis in *croquemort* and *draper* mutants (called *crq*$^{\Delta}$ and *drpr*$^{\Delta 5}$ respectively), and *E. coli* in *drpr*$^{\Delta 5}$ (Fig. 20A,B), bacteria adhesion to the hemocytes was not impaired in these mutants (Fig. 20C). This further supports a specific role of Eater as the main tethering receptor in Gram-positive bacteria phagocytosis. Moreover, *drpr*$^{\Delta 5}$ and *crq*$^{\Delta}$ hemocytes showed a wild-type like engulfment of latex beads (Fig. 20D),

further indicating a specific role of Eater in microbe uptake, likely via the recognition of a key bacterial surface determinant.

Discussion and Conclusions

NimC1 was initially identified as an antigen for the plasmatocyte specific monoclonal antibody P1. It belongs to the Nimrod gene family that has been implicated in the cellular innate immune response in *Drosophila* [43,44]. A previous study pointed to the importance of NimC1 in the phagocytosis of bacteria, since RNAi-mediated silencing of this gene resulted in decreased *S. aureus* uptake by plasmatocytes [14]. In the present work, we further re-evaluated the function of the NimC1 protein by using a novel null-mutant, revealing its precise role in hemocyte adhesion, proliferation and phagocytic ability.

By performing *ex vivo* spreading assays, we observed that the cell area of adherent *NimC1* null hemocytes was reduced compared to wild-type control, suggesting that NimC1 works as an adhesion molecule. Consistent with this observation, scanning electron microscopy on spread hemocytes of $NimC1^{1}$ mutants revealed a defect in lamellipodia extension. Spreading defects were also observed in $eater^{1}$ [16] and $NimC1^{1};eater^{1}$ hemocytes. Thus, two structurally related Nimrod receptors, NimC1 and Eater, are involved in lamellipodia extension and hemocyte adhesion. It will be interesting to analyse, in future work, the implications of NimC1 and Eater in hemocyte migration during metamorphosis or wound healing. Our results also indicate that Eater and NimC1 additively regulate hemocyte adherence. In contrast to *eater* deficient larvae, NimC1 is however not directly required for plasmatocyte sessility *in vivo*. Whether NimC1 contributes to hemocyte sessility through additional scaffold proteins has to be further investigated, even though the present evidence might favour a model where Eater is the only essential protein required for hemocyte sessility [16].

During larval development, the peripheral hemocyte population undergoes a significant proliferation, expanding by self-renewal [8,21]. Moreover, during these developmental stages, plasmatocytes are characterized by a dynamic behaviour, continuously exchanging between the sessile and circulating state. In 2011, Makhijani et al. provided evidence that plasmatocyte proliferation rate is higher in the hematopoietic pockets, where hemocytes cluster on the lateral side of the larval body [21]. At this location, sessile plasmatocytes are in contact with the endings of peripheral neurons, which are thought to provide a trophic environment to the blood cells. More recently, it has been shown that sensory neurons of the peripheral nervous system produce Activin-ß, which turned out to be an important factor in the regulation of hemocyte proliferation and adhesion [45]. By analysing the total number of hemocytes in third instar

NimC1^{1} or *eater*^{1} larvae, we observed that both Eater and NimC1 negatively regulate hemocyte counts in an additive manner. EdU incorporation experiments revealed that the higher hemocyte counts in *NimC1*^{1}*;eater*^{1} mutants were a consequence of an increased hemocyte proliferation rate. It is tempting to speculate that the higher proliferation rate is a secondary consequence of an adhesion defect. Indeed, adherent cells, notably when establishing contacts with other cells, are less proliferative, a process called "contact inhibition of proliferation" [46]. Future studies should address how Eater and NimC1 contribute to both adhesion and proliferation, and the direction of causality between these two processes remains to be disentangled. Like plasmatocytes, crystal cells increase in number during larval stages. However, crystal cell proliferation is not due to a self-renewal mechanism because mature crystal cells do not divide. Instead, a recent study has shown that new crystal cells originate from transdifferentiation of sessile plasmatocytes via a Notch-Serrate dependent process [26]. In the present study, we show that the *NimC1* deletion does not strongly impact crystal cell formation as both sessile and circulating crystal cell populations were only mildly affected in *NimC1* null larvae. Moreover, NimC1 does not affect the ability to differentiate lamellocytes and to encapsulate parasitoid wasp eggs.

NimC1 was initially identified as a phagocytic receptor, mediating the uptake of *S. aureus* bacteria [14]. Contrary to this study, our *ex vivo* phagocytosis assays using the *NimC1* deletion mutant revealed that the uptake of both Gram-positive and Gram-negative bacteria was not altered in *NimC1* null hemocytes. We hypothesized that the RNAi approach could have targeted other phagocytic receptors, revealing a stronger phenotype not observed in the single null mutant. Strikingly, phagocytosis of both bacteria types was severely impaired in *NimC1*^{1}*;eater*^{1} hemocytes, suggesting that both receptors contribute synergistically to phagocytosis of both Gram-negative and Gram-positive bacteria. At this stage, we cannot exclude that these receptors might indirectly regulate phagocytosis by controlling another receptor directly involved in bacterial recognition, although we judge this hypothesis unlikely. Consistent with our hypothesis, an RNAseq analysis of *eater* deficient versus wild-type hemocytes did not uncover any role of Eater in the regulation of other phagocytic receptors (data not shown).

Given the marked phagocytosis defect of the *eater* single mutant against *S. aureus*, the contribution of NimC1 was especially noticeable in the case of the Gram-negative bacterium *E. coli*. Our scanning electron microscopy approach revealed that NimC1 and Eater might contribute together to the early step of bacterial recognition, since *NimC1*^{1}*;eater*^{1} double mutants showed decreased bacterial adhesion. The involvement of NimC1 in *E. coli* binding is

consistent with previous *in vitro* work showing that native NimC1 binds bacteria [47]. Surprisingly, *NimC1*^{1} and *NimC1*^{1}*;eater*^{1}*,* but not *eater* deficient plasmatocytes, showed a significantly reduced ability to engulf latex beads and yeast zymosan particles. A recent study has also revealed a role of NimC1 in the phagocytosis of latex beads using an *in vivo* RNAi approach [48]. Thus, Eater and NimC1 have specific properties with regards to phagocytosis. It is interesting to address a parallel with the implication of two Nimrod receptors in bacteria tethering and docking, as shown for apoptotic cells clearance in *Drosophila melanogaster* [18,49]. Tethering receptors usually lack an intracellular domain and are involved in the binding to the dying cell. Docking receptors, instead, are subsequently required to activate intracellular signalling and mediate the internalization and degradation of the particle. In the fruit fly, a good example for tethering and docking receptors are SIMU/NimC4 and Draper, respectively [49]. A similar dichotomy exists in vertebrates, as Stabilin 2 and TIM-4 are classified as tethering receptors, whereas the integrins αVβ3 and αVβ5 are grouped as docking/signalling proteins [50,51]. The involvement and cooperation of two receptors of the Nimrod family in bacterial phagocytosis raised the possibility that they might contribute via different mechanisms: binding and internalization. Our current hypothesis is that Eater might work as the main tethering receptor, required for binding to specific motifs present on the bacterial surface. Moreover, given the wild-type engulfment of latex beads in *eater*, this receptor might be engaged specifically for phagocytosis of microbes. Indeed, the involvement of Eater in bacterial binding was already assessed in previous studies [35], and is consistent with our assays using live fluorescent bacteria and SEM experiments. On the contrary, NimC1 could function in the activation of the subsequent intracellular signalling, maybe as a subunit of a bigger macromolecular complex. We hypothesise that in the presence of cell wall bacterial determinants, such as peptidoglycan, lipopolysaccharide or teichoic acids, microbe phagocytosis can bypass the requirement of NimC1 by providing enough “eat me” signals to Eater. In contrast, the critical role of NimC1 in phagocytosis becomes visible with less immunogenic particles. This would explain why we do not observe any defects in the phagocytosis of *S. aureus* and *E. coli* in *NimC1* single mutant, but only with latex beads (i.e. particles without any bacterial motifs).

Future studies should address how Eater and NimC1 interact, the implication of other possible phagocytic receptors and characterization of their respective ligands. Collectively, our genetic analysis using compound mutants identifies NimC1 and Eater as two critical receptors involved in the initial step of phagocytosis, and notably adhesion to bacteria. While a plethora of receptors have been identified for their role in microbial phagocytosis in *Drosophila*, NimC1

and Eater appear to be the best candidates to directly recognize bacterial 'eat me' signals initiating phagocytosis. Our study also provides a valuable tool to better assess the role of phagocytosis during the immune response.

Materials and Methods

Drosophila stocks and methodology

All *Drosophila* stocks were maintained at 25°C on standard fly medium consisting of 6% cornmeal, 6% yeast, 0.62% agar, 0.1% fruit juice (consisting on 50% grape juice and 50% multifruits+multivitamin juice), supplemented with 10.6 g/L moldex and 4.9 ml/L propionic acid. Second instar (L2) larvae were selected 48-52 hours after egg laying (AEL), middle L3 larvae 72-90 hours AEL and third instar (L3) wandering larvae 110-120 hours AEL.

Wild type w^{1118} (BL5905) flies were used as controls, unless indicated otherwise. The following fly lines were used in this study:

	Details	Source
w^{1118}	BL5905	Bloomington
y^1w^{1118}		
$w^{1118};;eater^1$		[16]
$w^{1118};NimC1^1$		This study
$w^{1118};NimC1^1;eater^1$		This study
$w^{1118};;Hml\Delta dsred.nls$		[16]
$w^{1118};;eater^1Hml\Delta dsred.nls$		This study
$w^{1118};NimC1^1;Hml\Delta dsred.nls$		This study
$w^{1118};NimC1^1;eater^1Hml\Delta dsred.nls$		This study
$w^{1118};Hml\Delta Gal4>UAS\text{-}GFP$		[52]
$w^{1118};Hml\Delta Gal4,UAS\text{-}GFP;eater^1$		This study
$w^{1118};NimC1^1,Hml\Delta Gal4,UAS\text{-}GFP$		This study
$w^{1118};NimC1^1,Hml\Delta Gal4,UAS\text{-}GFP;eater^1$		This study
$yw,lzGal4>UAS\text{-}GFP$	BL6314	Bloomington
$yw,lzGal4,UAS\text{-}GFP;;eater^1$		This study
$yw,lzGal4,UAS\text{-}GFP;NimC1^1$		This study
$yw,lzGal4,UAS\text{-}GFP;NimC1^1;eater^1$		This study
$;;draper^{\Delta 5}$		[37,53]
croquemort		[54]
$PPO1^{\Delta},2^{\Delta},3^1$		[55]
UAS-NimC1-IR	VDRC 105799	Vienna *Drosophila* Resource Center
UAS-eater-IR	VDRC 30097	Vienna *Drosophila* Resource Center
$Relish^{E20}$		Described in [34]
;UAS-NimC1;		This study

Gene targeting of *NimC1*

Gene targeting of *NimC1* was performed as follows. The 5' and 3' homology arms, of 4.8kb and 3.7kb, respectively, were PCR amplified from genomic DNA. The 5' arm was inserted between *NotI* and *NheI* restriction sites, whereas the 3' arm was inserted between *SpeI* and *AscI* sites of the gene targeting vector pTV[Cherry]. A donor transgenic stock was generated by transformation of a starting *w1118* (BL5905) stock, and used for hsFLP and hs-I-SceI mediated gene targeting [19]. Using this method, we recorded a 1/2000 knockout efficiency of the F_2 progeny, i.e. 1/2000 offspring were bonafide *NimC1* knockouts.

The following primers were used for PCR genotyping and for testing the functional *NimC1* deletion by RT-PCR:

NAME	TARGET GENE	SEQUENCE
*eater*_F	*eater*	TAGGAGGTCATAAACGGTCA
*eater*_R	*eater*	CTCAAACGATTTGGACTTTG
*NimC1*_F	*NimC1*	AGTGTGCTCGTTATCTGGAA
*NimC1*_R	*NimC1*	GTTTCCCACTTTCTCGTACC
*NimC1*cDNA_F	*NimC1*	TCGCTTCAAGGACAACTCCC
*NimC*1cDNA_R	*NimC1*	ACACAGTCTCCGAATTGGCA

Hemocyte counting by flow cytometry

A BD Accuri C6 flow cytometer was used to analyse hemocyte numbers. For each genotype, 15 L2, 5 middle L3, or 5 L3 wandering third instar larvae containing the *HmlΔdsred.nls* marker were bled into 150 µL of Schneider's insect medium (Sigma-Aldrich) containing 1 nM phenylthiourea (PTU, Sigma). Before bleeding, larvae were vortexed in PBS 1X for one minute in order to detach sessile hemocytes [56]. 100 µL of hemocyte suspension was analysed by flow cytometry. Hemocytes were first selected from debris by plotting FSC-A against SSC-A on a logarithmic scale in a dot plot. Cells were then gated for singlets by plotting FSC-H versus FSC-A. w^{1118} and w^{1118};;*HmlΔdsred.nls* larvae were used to define the gates for hemocyte population, using a FL2 detector.

Hemocyte size measurement of free-floating cells

Third instar (L3) wandering larvae were bled in 1X PBS without calcium and magnesium, supplemented with EDTA 5mM. Invitrogen™ Tali™ Image-based Cytometer machine was used to measure hemocytes size in suspension of more than 7 000 cells per genotype.

***Ex vivo* larval hemocyte phagocytosis assays**

1) *Ex vivo* phagocytosis assay of *E. coli* and *S. aureus* was performed using *E. coli* and *S. aureus* AlexaFluor™488 BioParticles™ (Invitrogen), following manufacturer's instructions. L3 wandering larvae carrying the *HmlΔdsred.nls* hemocytes marker were bled into 150 µL of Schneider's insect medium (Sigma-Aldrich) containing 1 µM phenylthiourea (PTU, Sigma-Aldrich). The hemocyte suspension was then transferred to 1.5 mL low binding tubes (Eppendorf) and 2 x 10^7 AlexaFluor™488 bacteria BioParticles™ were added. The samples were incubated at room temperature for 30 or 60 minutes to enable phagocytosis, and then placed on ice in order to stop the reaction. The fluorescence of extracellular particles was quenched by adding 0.4% trypan blue (Sigma-Aldrich) diluted 1/3. Phagocytosis was quantified using a flow cytometer (BD Accuri C6, USA) in order to measure the fraction of cells phagocytosing, and their fluorescent intensity. w^{1118} larvae and *HmlΔdsred.nls* larvae with or without bacterial particles were used to define the gates for hemocytes and the thresholds for phagocytosed particle emission. The phagocytic index was calculated as follows:

$$\textbf{Fraction of hemocytes phagocytosing (f)} = \frac{[number\ of\ hemocytes\ in\ fluorescence\ positive\ gate]}{[total\ number\ of\ hemocytes]}$$

$$\textbf{Phagocytic index (PI)} = [Mean\ fluorescence\ intensity\ of\ hemocytes\ in\ fluorescence\ positive\ gate]\ x\ f$$

2) *Ex vivo* phagocytosis assays of *S. marcescens*, *S. epidermidis*, and *M. luteus* was performed as follows. Bacterial strains and labelling bacteria with fluorescein isothiocyanate (FITC) are described in [47]. The stocks of *S. marcescens* (Szeged Microbial Collection, University of Szeged, Szeged, Hungary; SzMC 0567), *S. epidermidis* (SzMC 14531), and *M. luteus* (SzMC 0264) were used. Bacteria were conjugated by FITC as described by Zsámboki et al. Briefly, 10 ml of bacterial culture (OD_{600}=1.5) was heat-inactivated in PBS and the cell pellet was re-suspended in 10 mL of 0.25 M carbonate-bicarbonate buffer pH 9.0. Fluorescein isothiocyanate (FITC) 0.5 mg, dissolved in 100 µL DMSO (Sigma) was added to the heat inactivated bacteria, rotated overnight at 4 ºC and washed eight times with PBS. The FITC labelled bacteria were re-suspended, centrifuged at 11200 x g, the pellet was resuspended to a final concentration of 10%, sodium azide was added as a preservative (0.1%) and the samples

were kept at 4°C until use. Bacteria were washed 5x with PBS prior to the phagocytosis assay. The phagocytic activity of hemocytes was assayed with a protocol similar to [6]. Hemocytes were isolated from third instar larvae at room temperature into Shields and Sang M3 insect medium (Sigma) containing 5% fetal-calf serum (Gibco) supplemented with 1-phenyl-2-thiourea (Sigma) to prevent melanization. A total of 2-3x10.5 hemocytes were incubated with 5-6 x 10^6 heat-killed, FITC labeled bacteria at room temperature for 40 min in the wells of round bottomed microtiter plates (Gibco) in 100 µL. The fluorescence of extracellular bacteria was quenched by the addition of Trypan blue to the cells in 0.2% final concentration shortly before the actual measurement. The fluorescence intensity of phagocytosed FITC-labeled bacteria was analyzed with a FACS Calibur equipment (Beckton Dickinson). Phagocytic index was calculated as mentioned above.

3) Phagocytosis of green fluorescent 1 µm latex beads (Sigma-Aldrich) and AlexaFluor™488 Zymosan BioParticles™ (Invitrogen) was performed following the same procedure described in 1), with the exception that hemocytes without the *HmlΔdsred.nls* marker were used. 1 x 10^5 Zymosan BioParticles™ and 0.2 µg of latex beads were added to each sample.

Given the difference in hemocyte numbers per larva between the genotypes, we bled 6 w^{1118} (*BL5905*) larvae, 4 $eater^1$ and $NimC1^1$ larvae, and 3 $NimC1^1;eater^1$larvae.

Proliferation assays

Cell proliferation was assessed by 5-ethynyl-2′-deoxyuridine (EdU) labelling. Second instar, or middle L3 larvae were fed at 29°C with 1 mM 5-ethynyl-2 deoxyuridine (EdU) in fly food for 4 hours. Larvae were bled individually in 30 µL Schneider medium (Gibco) containing 1 nM phenylthiourea (PTU, Sigma). Hemocytes were allowed to settle for 30 minutes before being fixed in 4% paraformaldehyde PBS. Click-iT™ EdU Imaging Kit (Invitrogen) was used to stain *HmlΔdsred.nls* hemocyte populations. Cells were finally stained with 1/15000 dilution of 4’,6- diamidino-2-phenylindole DAPI (Sigma) and mounted in anti-fading agent Citifluor AF1 (Citifluor Ltd). The proliferation rate was determined by counting the number of EdU positive cells over the whole *HmlΔdsred.nls* hemocyte population. At least 6 animals were analysed per genotype.

Scanning electron microscopy (SEM)

Samples for SEM were prepared as follows. Six wandering third instar larvae were bled into 50 µL of Schneider’s insect medium (Sigma-Aldrich) containing 1µM phenylthiourea (PTU,

Sigma-Aldrich). The collected hemolymph was incubated on a glass coverslip for 20 minutes for spreading assay, or 30 minutes with bacteria for phagocytosis assay, before being fixed for one hour with 1.25% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Samples were then washed in cacodylate buffer (0.1 M, pH 7.4), fixed again in 0.2% osmium tetroxide in the same washing buffer and then dehydrated in graded alcohol series. Samples underwent critical point drying and Au/Pd coating (4 nm). Scanning electron micrographs were taken with a field emission scanning electron microscope Merlin, Zeiss NTS, Germany.

Transmission electron microscopy (TEM)

Third instar wandering larvae were bled in 50 µL of Schneider's insect medium (Sigma-Aldrich) containing 1µM phenylthiourea (PTU, Sigma-Aldrich). The collected hemolymph was incubated with bacteria on a glass coverslip for one hour before being fixed for two hours with 2% paraformaldehyde + 2.5% glutaraldehyde in 0.1M phosphate buffer, pH 7.4. Samples were then washed in cacodylate buffer (0.1M, pH 7.4), fixed again in 1% osmium tetroxide and potassium ferrocyanide 1.5% in cacodylate buffer. After washes in distilled water, samples were stained in 1% uranyl acetate in water, washed again, and then dehydrated in graded alcohol series (2X50%, 1X70%, 1X90%, 1X95%, 2X100%). Embedding was performed first in 1:1 Hard EPON and ethanol 100%, and afterwards in pure EPON, before being embedded on coated glass slides and placed at 60°C overnight. Images were acquired with a FEI Tecnai Spirit 120 kV.

Binding assay with live fluorescent bacteria

Cytochalasin D treatment. L3 wandering larvae were bled into 120 µL of Schneider's insect medium (Sigma-Aldrich) containing 1 µM phenylthiourea (PTU, Sigma-Aldrich). Hemocytes were allowed to adhere on the glass slide for one hour before being treated for another 60 minutes with 1 µM of Cytochalasin D. After drug treatment, hemocytes were incubated directly on the slide with live fluorescent *S. aureus* or *E. coli* bacteria always in the presence of Cytochalasin D for 60 minutes. After fixation in 4% paraformaldehyde PBS, rhodamine phalloidin staining (Molecular Probes™) was performed. Finally, cells were stained with 1/15000 dilution of 4',6- diamidino-2-phenylindole DAPI (Sigma) and mounted in anti-fading agent Citifluor AF1 (Citifluor Ltd.).

Phagocytosis inhibition by cold temperature. L3 wandering larvae were bled into cold Schneider's insect medium (Sigma-Aldrich) containing 1 µM phenylthiourea (PTU, Sigma-Aldrich) on a previously chilled glass slide. After larva bleeding, hemocytes and bacteria were

incubated directly on the pre-chilled slide, in cold Schneider's medium, on ice for 60 minutes. Fixation and staining procedures were performed as described above.

Fluorescent bacteria. The *E. coli* GFP strain was obtained by transforming *E. coli* K12 with a synthetic sfGFP coding sequence cloned in a pBAD backbone (ThermoFisher) by Gibson assembly. sfGFP induction was obtained by growing the bacteria in LB + 0.1% arabinose overnight prior to the binding assay. The *S. aureus* GFP strain is described in [57].

Hemocyte phalloidin staining and cell area measurement

Five wandering third instar larvae were bled on a microscope slide into 120 µL of 1X PBS containing 1 µM phenylthiourea (PTU, Sigma-Aldrich). The hemocytes were then allowed to adhere for 30 minutes, before being fixed in 4% paraformaldehyde PBS. Phalloidin staining was performed with diluted 1/100 AlexaFluor488- or rhodamine-phalloidin (Molecular Probes™). Finally, cells were stained with a 1/15000 dilution of 4',6- diamidino-2-phenylindole DAPI (Sigma) and mounted in anti-fading agent Citifluor AF1 (Citifluor Ltd.). Samples were imaged with an Axioplot Imager.Z1 Zeiss coupled to an AxioCam MRm camera (Zeiss).

For cell area measurements, hemocytes were captured with a 20x objective on GFP, RFP and DAPI channels. Individual images were then loaded into a CellProfiler pipeline (www.cellprofiler.org). In order to define the cell area, cell nuclei were first detected using data from the DAPI channel. Cell limits were then defined by expanding the nuclei signal to the edges of the GFP channel. Cell areas were computed from this segmentation analysis, and cell area of 750 cells of each genotype was quantified.

Hemocytes visualization through larva cross sectioning

Third instar larvae of the indicated genotypes were fixed in 4% paraformaldehyde PBS for 48 hours at 4°C. Afterwards, larvae were embedded using OCT medium in a Tissue-Tek cryomolds (Sakura). Transverse sections of 4 to 5 µm thickness were cut using Leica CM1959 cryostat. Finally, sections were fixed again for 15 minutes in 4% paraformaldehyde PBS, prior to rhodamine phalloidin (Molecular Probes™) staining. Samples were imaged with an Axioplot Imager.Z1 Zeiss coupled to an AxioCam MRm camera (Zeiss).

Crystal cell counting methods

At least ten third instar larvae were heated in 1 mL of phosphate-buffered saline (PBS) at 67°C for 20 minutes in eppendorf tubes. For quantification analysis, black puncta were counted in

the posterior-most segments A6, A7 and A8. Pictures were taken with a Leica DFC300FX camera and Leica Application Suite right after heating.

For quantification of crystal cells by flow cytometry, we crossed wild-type or mutant *lzGal4>UAS-GFP* flies with the corresponding *HmlΔdsred.nls* w^{1118} or mutant flies. Larvae form the resulting offspring were used to determine the number of crystal cells (*lzGal4>UAS-GFP*) and the ratio of crystal cells among the total hemocyte population (*lzGal4>UAS-GFP* / *HmlΔdsred.nls*). Four larvae of each genotype were bled into 150 µL 1X PBS containing 1µM phenylthiourea (PTU, Sigma-Aldrich) and 0.1% paraformaldehyde to block crystal cell rupture. 75 µL of the hemocyte suspension was analysed by flow cytometry. Hemocytes were first selected from debris by plotting FSC-A against SSC-A on a logarithmic scale in a dot plot. Cells were then gated for singlets by plotting FSC-H versus FSC-A. FL1 and FL2 detectors were used for *lzGal4>UAS-GFP* and *HmlΔdsred.nls* events, respectively.

Wounding experiment

Wandering third-instar larvae were pricked dorsally near the posterior end of the animal, using a sterile needle (diameter ~5 µm). Pictures of melanised larvae were taken 20 minutes after pricking, with a Leica DFC300FX camera and Leica Application Suite.

Wasp Infestation and quantification of fly survival to *L. boulardi* infestation

For wasp infestations experiments, 30 synchronized second instar (L2) larvae were placed on a pea-sized mound of fly food within a custom-built wasp trap in the presence of three female *Leptopilina boulardi* (strain NS1c, described in [58]) for 2 hours. Quantification of fly survival was performed as follows. Parasitized larvae were kept at room temperature and scored daily for flies or wasps emergence. The number of eclosed flies and wasps was subtracted of the initial number of exposed larvae and set as dead larvae/pupae. Pictures of melanised eggs were taken with a Leica DFC300FX camera and Leica Application Suite.

Infection experiments and qRT-PCR

Systemic infections (septic injuries) were performed by pricking third instar larvae dorsally near the posterior end of the animal using a thin needle previously dipped into a concentrated pellet (OD_{600} ~200) of bacteria. After septic injury, larvae were incubated at 29°C. After 4 hours, the animals were collected, and total RNA extraction was performed using TRIzol reagent (Invitrogen). RNA quality and quantity were determined using a NanoDrop ND-1000 spectrophotometer and 500 ng of total RNA was used to generate cDNA using SuperScript II

(Invitrogen, Carlsbad, California, United States). Quantitative PCR was performed on cDNA samples using the LightCycler 480 SYBR Green Master Mix (Roche). Expression values were normalized to *RpL32*.

Statistical analysis

Experiments were repeated at least three times independently and values are represented as the mean ± standard deviation (SD). Data were analysed using GraphPad Prism 7.0. *p*-values were determined with Mann-Whitney tests, unless indicated otherwise. For phagocytic index measurement experiments, data successfully passed a Shapiro-Wilk normality test (alpha=0.05, n=9), so that we could assume that samples follow Gaussian distribution. Therefore, significance tests were performed using Students *t* test.

Authors contributions

CM and BL conceived and designed the project. CM, AJB, and ER contributed to the generation of the *NimC1*^{1} mutant and other tools used in this study. JD and IA performed the wasp experiments, and EK and IA did the phagocytosis assay with *S. epidermidis*, *M. luteus*, and *S. marcescens*. CM performed all the other experiments of the study. CM and BL wrote the paper.

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Conflicts of interest

The authors declare no conflicts of interest.

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Figures

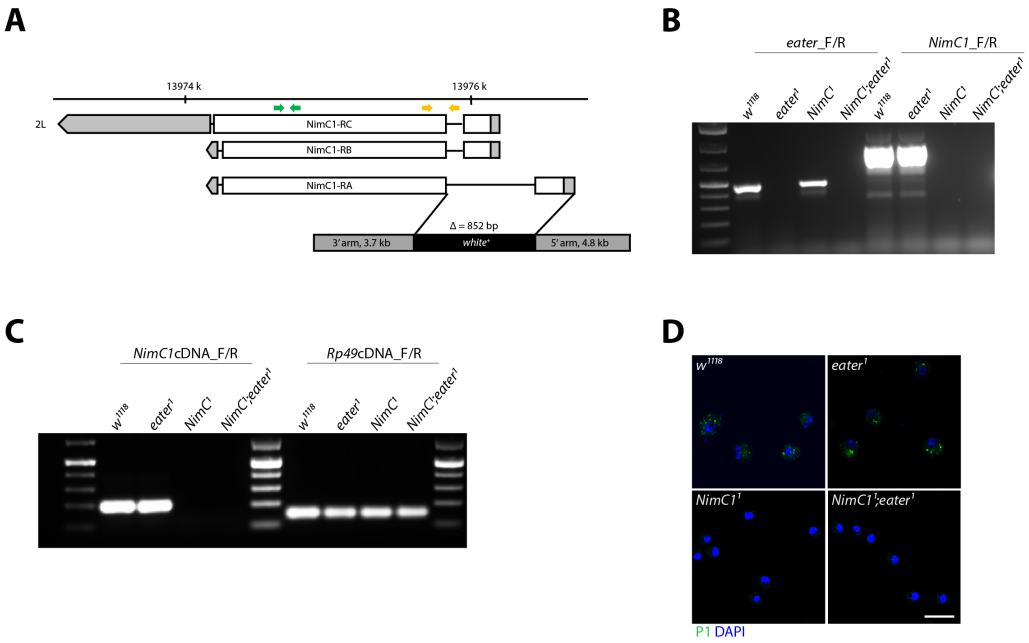

Figure 10. Gene targeting and deletion of *NimC1*.

(A) *NimC1* gene deletion by homologous recombination. The *NimC1* gene is located on the left (L) arm of chromosome 2 and it encodes three isoforms. White and grey boxes represent exons and UTR regions, respectively. Eye colour was transformed from white to red by the white^{+} marker. Yellow and green arrows represent, respectively, the approximate location of primers used in (B) and (C). **(B)** PCR genotyping confirming the targeted deletion of the *NimC1* gene, whereas the eater locus was not affected. **(C)** RT-PCRs confirming functional deletion of *NimC1*. **(D)** NimC1 (P1) staining (green) of third instar larval hemocytes from the indicated genotypes. Cell nuclei are shown in DAPI (blue). The immunostaining was performed as previously described in [59]. Scale bar: 20 µm.

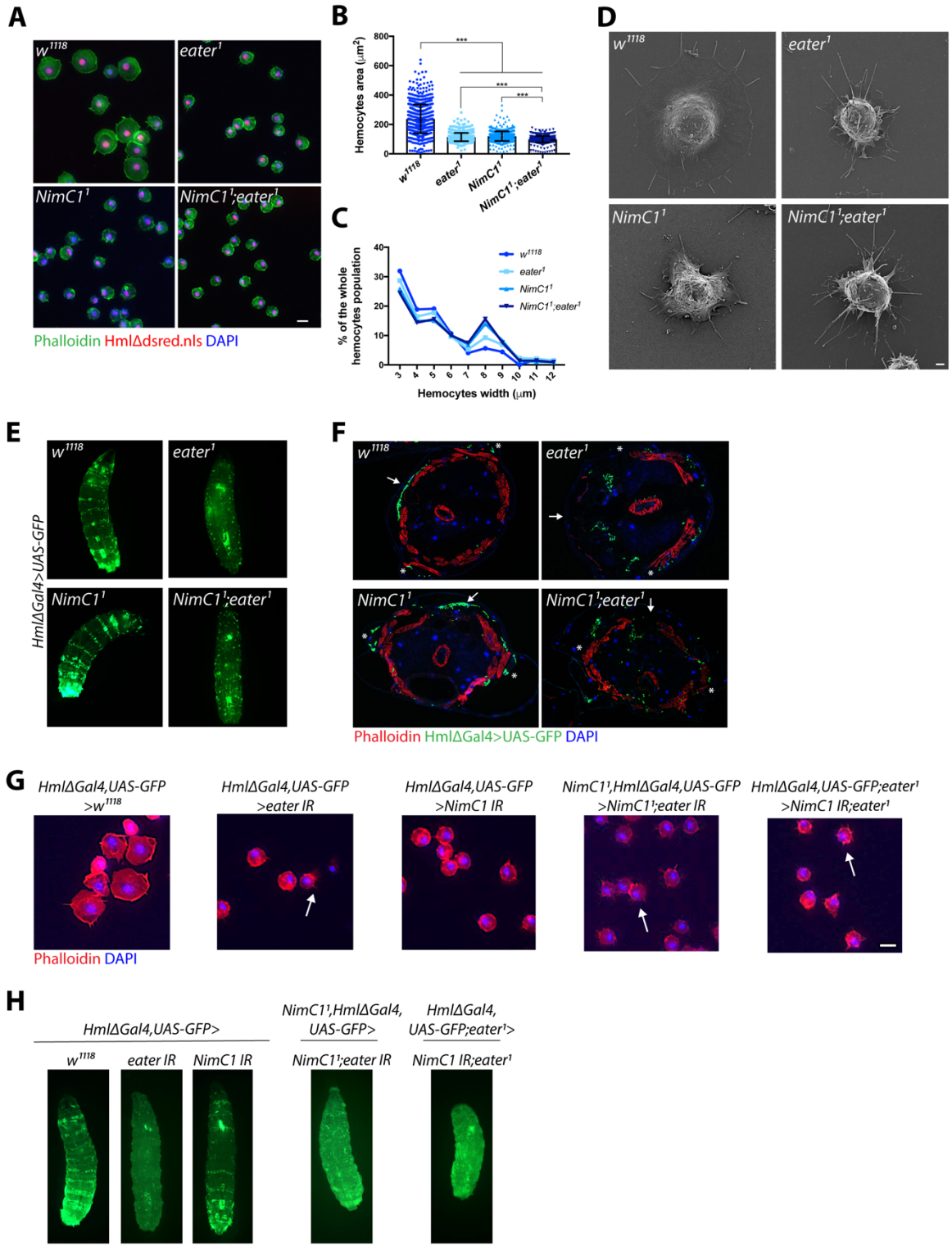

Figure 11. *NimC1[1]* hemocytes show spreading defects *in vitro*.

(A) Representative images of fixed hemocytes from *w[1118]*, *eater[1]*, *NimC1[1]* and *NimC1[1];eater[1]* L3 wandering larvae combined with *HmlΔdsred.nls* marker (red). Hemocytes of the indicated genotypes were extracted by larval bleeding, allowed to spread for 30 minutes on a glass slide, and stained with AlexaFluor™488 phalloidin (green). Scale bar: 10 μm. **(B)** Mean cell area

quantification of fixed *HmlΔdsred.nls* hemocytes spread for 30 minutes on slides and stained with AlexaFluor488 phalloidin. Cell area of 750 cells was quantified using the CellProfiler software. **(C)** Size distribution of free floating hemocytes from w^{1118}, $eater^{1}$, $NimC1^{1}$ and $NimC1^{1}$;$eater^{1}$ L3 wandering larvae. Hemocyte size was measured with TALI imaged-based cytometer directly after larval bleeding of more than 7 000 cells per genotype. **(D)** Representative scanning electron microscopy images of spread hemocytes from the indicated genotypes of L3 wandering larvae. Scale bar: 1 µm. **(E)** Whole larva images of w^{1118}, $eater^{1}$, $NimC1^{1}$ and $NimC1^{1}$;$eater^{1}$ third instar larvae specifically expressing *UAS-GFP* in plasmatocytes driven by *Hml*Δ-*GAL4*. The dorsal side of the animal is shown. **(F)** Cross sections of the indicated genotypes from L3 wandering larvae combined with *HmlΔGal4>UAS-GFP* (green). Rhodamine phalloidin staining (red) was performed after larva cross sectioning. Cell nuclei are shown in DAPI (blue). White arrows and asterisk indicate dorsal and lateral side of the animal, respectively. **(G)** Representative images of fixed hemocytes from the indicated genotypes of L3 wandering larvae, stained with rhodamine phalloidin (red). Cell nuclei were stained with DAPI (blue). Arrows indicate the presence of filopodia in the corresponding genotype, found also in the eater deletion mutants. Scale bar: 10 µm. **(H)** Whole larva images of third instar larvae of the indicated genotypes, specifically expressing GFP in plasmatocytes driven by *Hml*Δ-*GAL4*. The dorsal side of the animal is shown. *** $p<0.001$ by Mann-Whitney tests.

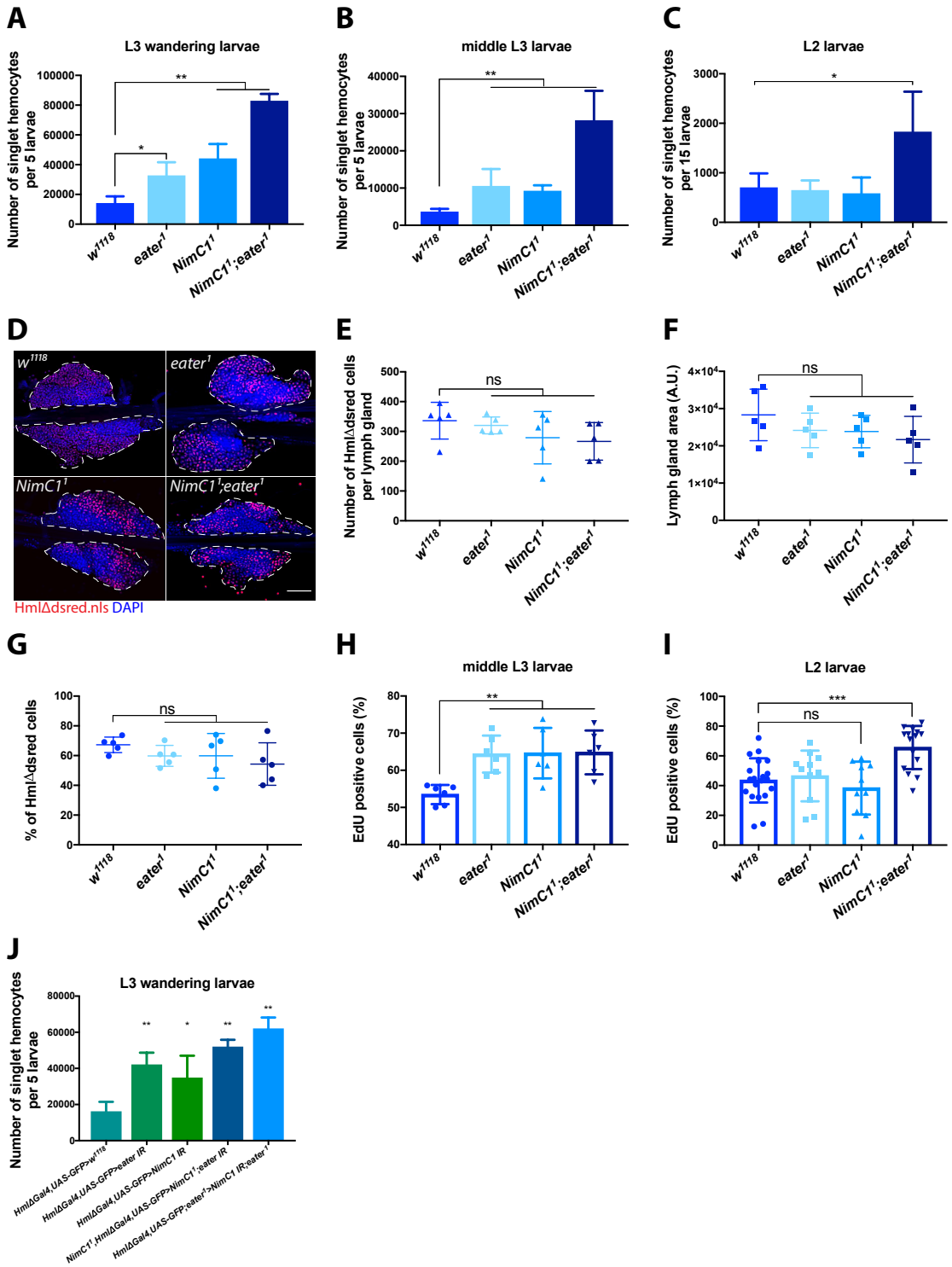

Figure 12. *NimC1*^{1} and *NimC1*^{1}*;eater*^{1} larvae give rise to a higher number of hemocytes. Number of singlet peripheral hemocytes per 5 L3 wandering **(A)**, 5 middle L3 **(B)**, and 15 L2 **(C)** larvae of *w*^{1118}, *eater*^{1}, *NimC1*^{1}, *NimC1*^{1}*;eater*^{1} combined with *HmlΔdsred.nls*. In (A-C) data are represented as mean ± SD from 5 independent experiments. **(D)** Representative confocal images of dissected lymph glands from *w*^{1118}, *eater*^{1}, *NimC1*^{1}, *NimC1*^{1}*;eater*^{1} third instar larvae combined with *HmlΔdsred.nls* hemocyte marker (red). Lymph gland primary

lobes are shown and boundaries are delimited by a white dashed line. Cell nuclei were stained with DAPI (blue) after paraformaldehyde fixation. Scale bar: 50 µm. Images were acquired with Zeiss LSM700 confocal microscope. **(E)** Absolute number of *HmlΔdsred.nls* cells in primary lymph gland lobes of the indicated genotypes. **(F)** Lymph gland area quantification in the indicated genotypes, performed by using ImageJ software tool. A.U.=arbitrary units. **(G)** Percentage of *HmlΔdsred.nls* cells upon DAPI positive cells in primary lymph gland lobes of the indicated genotypes. Five primary lymph gland primary lobes per genotypes were analysed in (E-G). Percentage of EdU positive cells upon *HmlΔdsred.nls* cells in middle L3 **(H)** and L2 larval **(I)** stage of w^{1118}, $eater^{1}$, $NimC1^{1}$, $NimC1^{1}$;$eater^{1}$. A number of at least 6 animals was used for each genotype. **(J)** Number of singlet peripheral hemocytes per 5 L3 wandering larvae of the indicated genotypes. Data are represented as mean ± SD from 5 independent experiments. * p<0.05, ** p<0.01, *** p<0.001 by Mann-Whitney test.

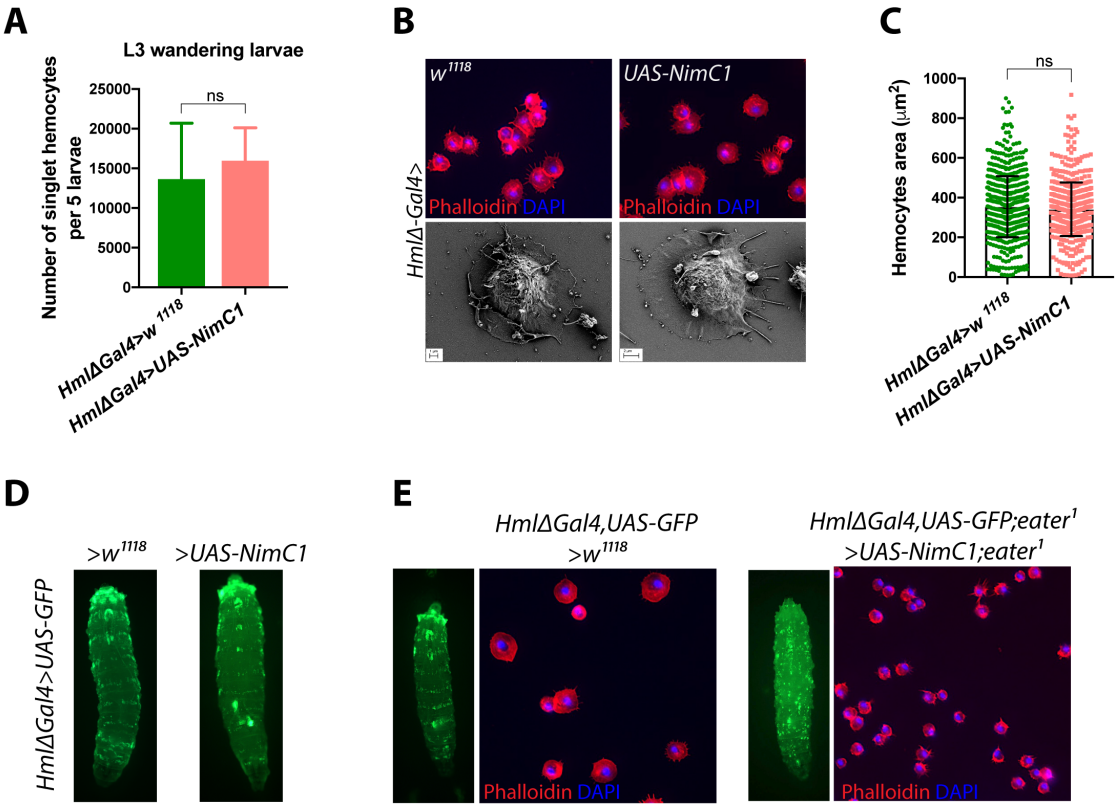

Figure 13. *NimC1* overexpression does not alter hemocyte number and adhesive properties. (A) Number of singlet peripheral hemocytes in third instar wandering larvae is not affected upon *NimC1* overexpression. Results are represented as a sum of 5 animals with the indicated genotypes. Data are represented as mean ± SD from 5 independent experiments. **(B)**

Upper panel: Representative images for fixed hemocytes from *HmlΔGal4>w*^{1118} and *HmlΔGal4>UAS-NimC1* L3 wandering larvae stained with rhodamine phalloidin (red). Cell nuclei are shown in DAPI (blue). Bottom panel: scanning electron micrographs on spread hemocytes from *HmlΔGal4>w*^{1118} and *HmlΔGal4>UAS-NimC1* of L3 wandering larvae. (**C**) Mean cell area quantification of fixed hemocytes of the indicated genotypes, spread for 30 minutes on slides and stained with AlexaFluor488 phalloidin. Cell area of 750 cells was quantified using the CellProfiler software. **(D)** Whole larva imaging of *HmlΔGal4,UAS-GFP>w*^{1118} and *HmlΔGal4,UAS-GFP>UAS-NimC1* shows no major difference in hemocyte localization pattern and adherence when NimC1 is specifically overexpressed in hemocytes. The dorsal side of the animal is shown. **(E)** Whole larva imaging and spreading assay showing the absence of rescue when overexpressing NimC1 in an eater mutant background. Data in (A) and (C) were analysed by Mann-Whitney test. ns: not significant.

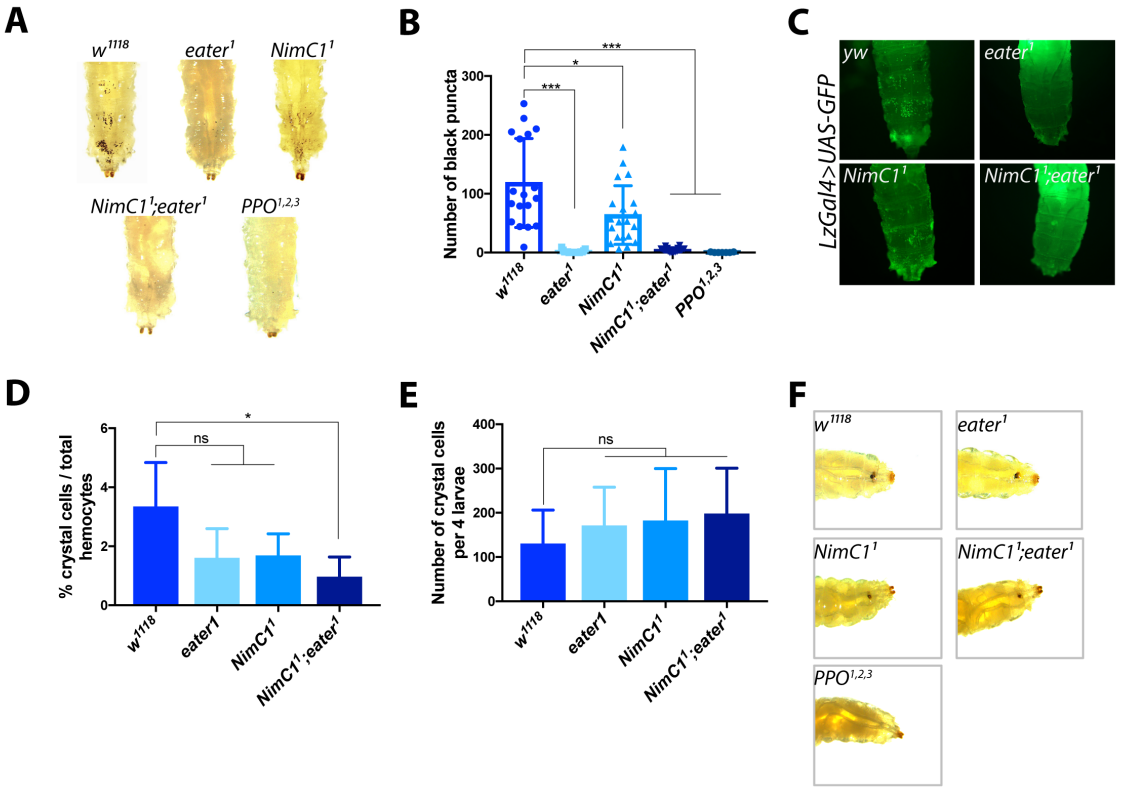

Figure 14. Sessile and circulating crystal cell populations are mildly affected in *NimC1*^{1} larvae. (A) Heating larvae induces the spontaneous activation of the prophenoloxidase zymogen within crystal cells, leading to their blackening [60]. Consequently, the population of crystal cells attached under the cuticle becomes visible as black puncta. Sessile crystal cell numbers were mildly reduced in *NimC1*^{1} larvae, while being almost completely absent in *eater*^{1}

and *NimC1[1];eater[1]* larvae. Shown are representative images of *w[1118], eater[1], NimC1[1]* and *NimC1[1];eater[1]* L3 wandering larvae after heat treatment at 67°C for 20 minutes. **(B)** Black puncta count from the three posterior-most segments of heated w^{1118}, $eater^1$, $NimC1^1$ and *NimC1[1];eater[1]* third instar larvae. A number of at least 18 animals was used for each genotype. **(C)** In-vivo imaging using the *lzGal4>UAS-GFP* crystal cell marker confirmed the previous observations. Shown is the dorsal view of the five posterior-most segments in yw, $eater^1$, *NimC1[1]* and *NimC1[1];eater[1]* L3 wandering larvae, previously combined with the crystal cell lineage marker *lzGal4>UAS-GFP*. **(D-E)** Flow cytometry counting of *lzGal4>UAS-GFP* positive cells revealed a *wild-type* number of crystal cells in *NimC1* and *eater* deficient L3 wandering larvae (D), and a moderately decreased ratio of crystal cells over the total hemocyte population (E), pointing to a mild defect in sessility but not in the general ability to differentiate crystal cells. Data are represented as mean ± SD from 5 independent experiments. (**F**) Melanization response to epithelial wounding is wild-type like in *eater* and *NimC1* mutants larvae. Representative images of melanized larvae were acquired 20 minutes after pricking. In (A) and (F) *PPO[1,2,3]* mutant larvae were used as negative control. * $p<0.05$, *** $p<0.001$, by Mann-Whitney tests. ns: not significant.

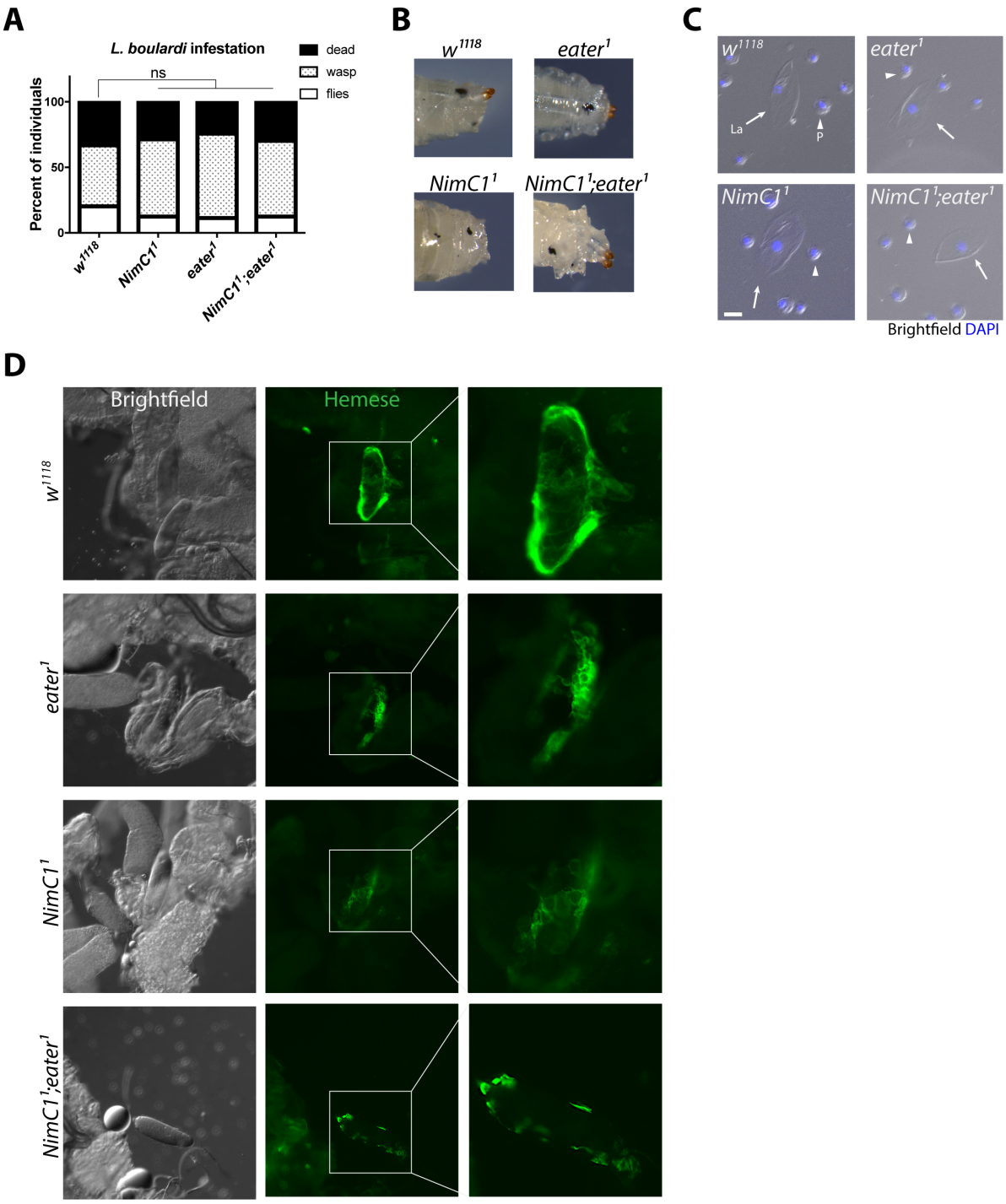

Figure 15. *NimC1* mutants do not show any major encapsulation defects after wasp infestation.

(A) Quantification of emerging *D. melanogaster* adult, *L. boulardi* wasp, and dead animals following parasitization with the parasitoid wasp *L. boulardi*. Data are shown as a sum of three experiments, with a total of 90 animals for each genotype. Data were analysed using Chi-square statistical test (p-value > 0.05). ns: not significant. **(B)** Shown are representative images of melanised wasp eggs in w^{1118} control and mutants, 70 hours after *L. boulardi* infestation. **(C)**

Lamellocyte differentiation is observed in *eater* and *NimC1* mutants upon wasp infestation. Representative images showing circulating hemocytes 70 hours after *L. boulardi* infestation. Arrows and arrowheads indicate hemocytes with lamellocytes (La) and plasmatocytes (P) morphology, respectively. Cell nuclei were stained with DAPI (blue) Scale bar: 20 µm. **(D)** Representative images showing early wasp egg recognition by peripheral plasmatocytes (hemocytes stained with anti-Hemese antibody [61], green) 20 hours after infestation. Hemocytes of both *wild-type* and *eater* and *NimC1* mutants attached to the eggs. However, single and double mutants plasmatocytes adhere with slightly decreased spreading ability compared to the *wild-type*.

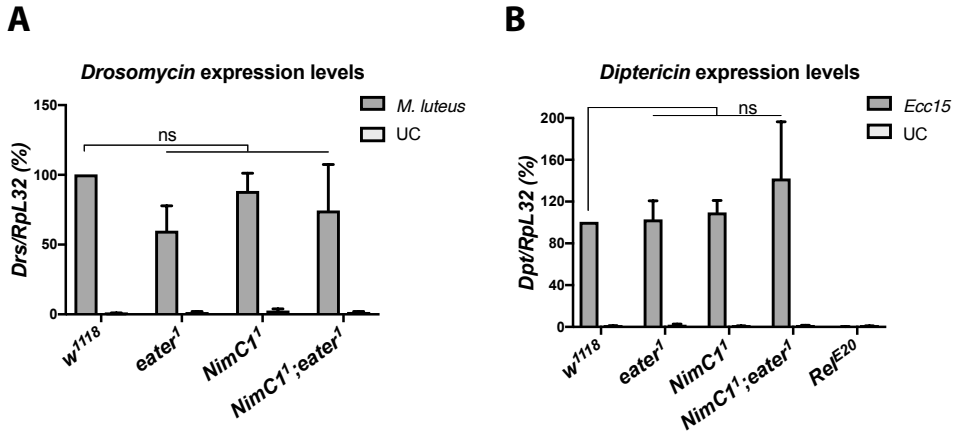

Figure 16. NimC1 is not involved in humoral immunity.

Expression levels of *Diptericin* **(A)** and *Drosomycin* **(B)** relative to *RpL32* in w^{1118}, $eater^{1}$, $NimC1^{1}$ *and* $NimC1^{1}$;$eater^{1}$ third instar larvae. Total RNA from infected animals was extracted 4 hours after *Ecc15* or *M. luteus* septic injury. UC= unchallenged controls. The Imd pathway mutant *Relish* (Rel^{E20}) was used as an immune-deficient control in (A). Data are represented as mean ± SD from 3 independent experiments ns: not significant, by Mann-Whitney test.

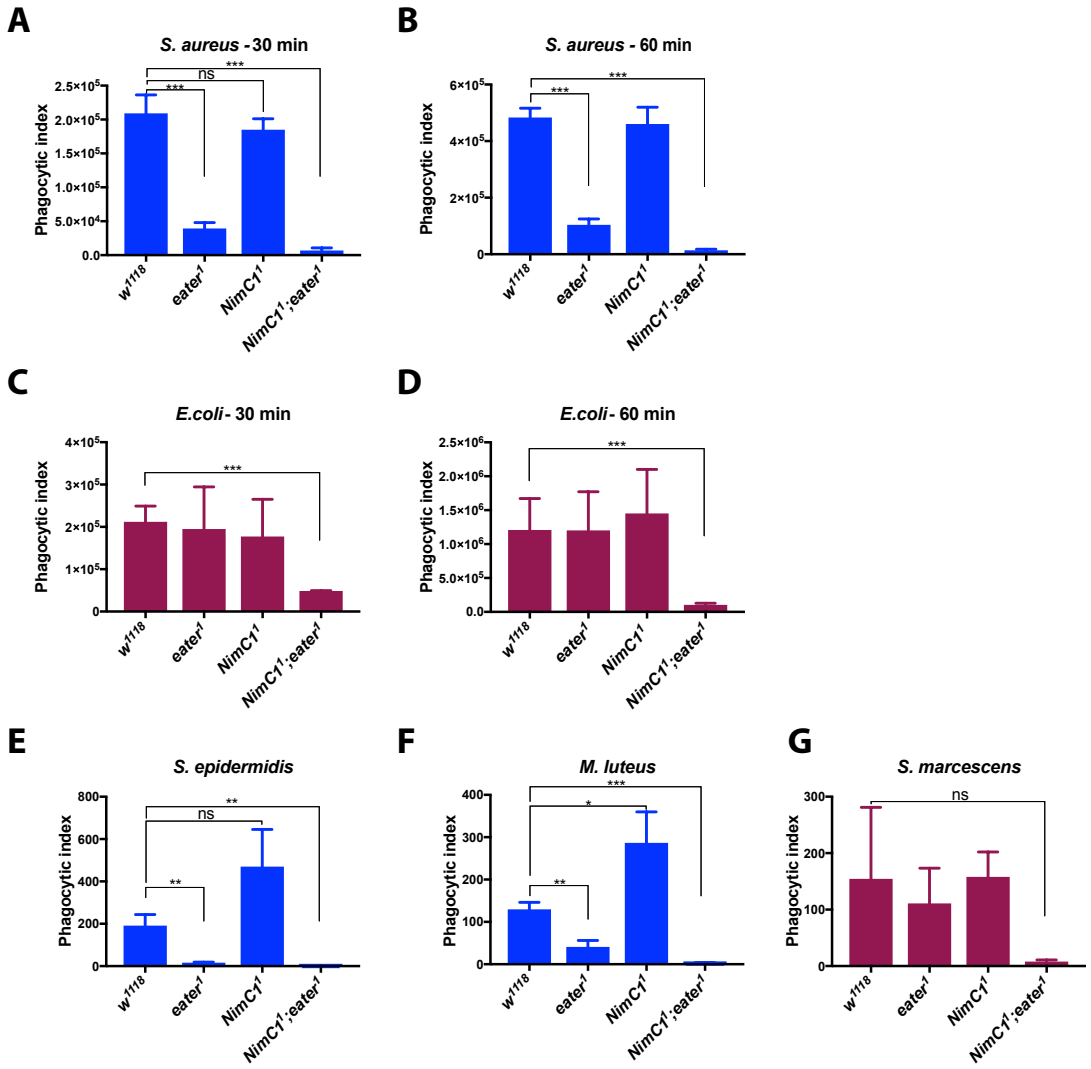

Figure 17. NimC1 contributes with Eater to phagocytosis of Gram-positive and Gram-negative bacteria.

Ex vivo phagocytosis assay using *S. aureus* **(A-B)** and *E. coli* **(C-D)** AlexaFluor™488 BioParticles™ (Invitrogen). *HmlΔdsred.nls* hemocytes from third instar wandering larvae were incubated with the particles for 60 (A,C) or 30 (B,D) minutes at room temperature. In (A-D) data are represented as mean ± SD from 4 independent experiments. *Ex vivo* phagocytosis assay using the Gram-positive *S. epidermidis* **(E)**, *M. luteus* **(F)**, and Gram-negative *S. marcescens* **(G)** bacteria. Bacteria were first heat-inactivated and subsequently labelled with fluorescein isothiocynate (FITC). Hemocytes from third instar wandering larvae of the indicated genotypes were incubated with the bacteria for 40 minutes at room temperature. In (E-F) data are represented as mean ± SD from 3 independent experiments. In (A-G), phagocytosis was quantified by flow cytometry, and the fluorescence of extracellular particles quenched by adding trypan blue. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, by Student t tests. ns: not significant.

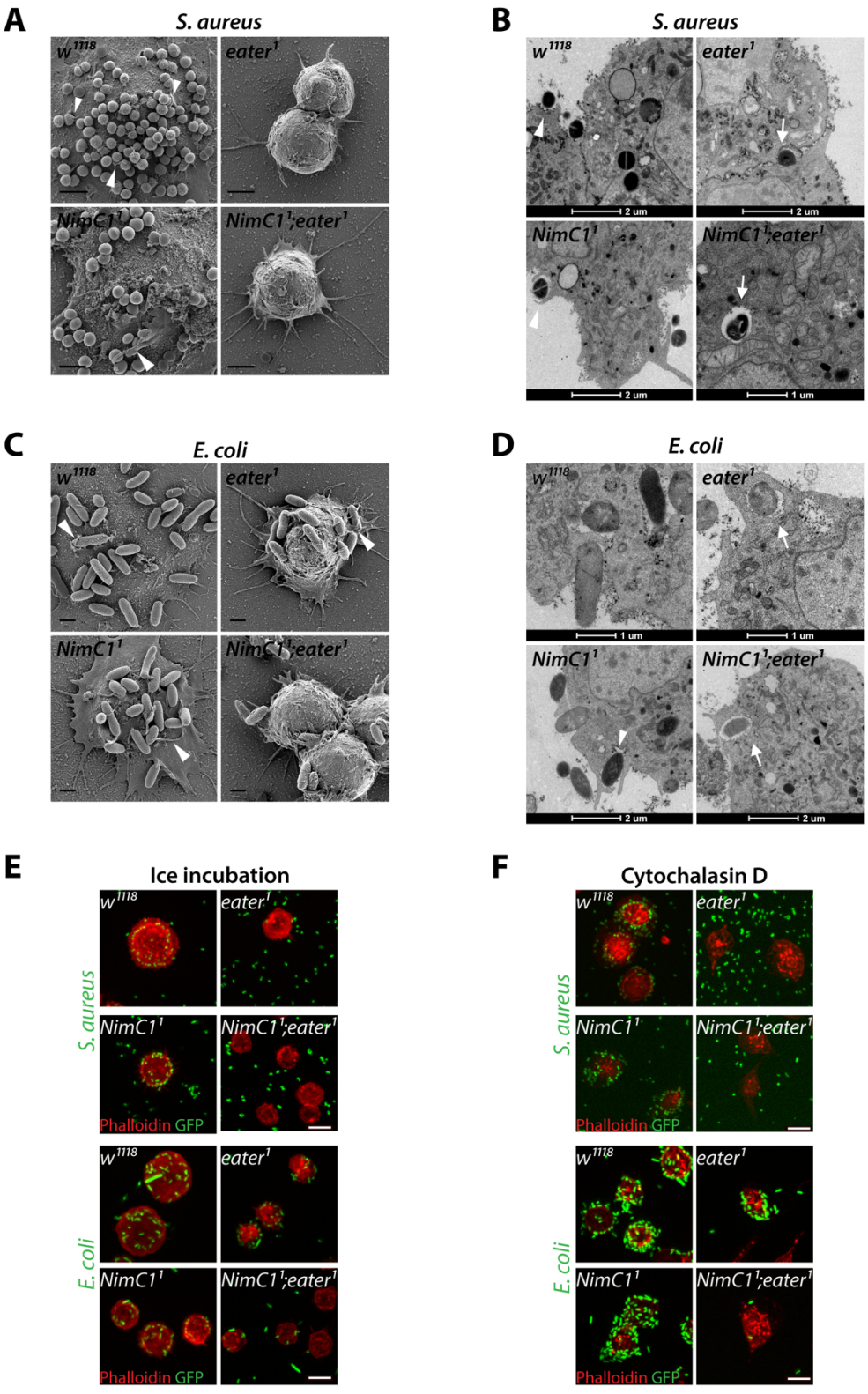

Figure 18. *eater*^{1} and *NimC1*^{1}*;eater*^{1} hemocytes show bacteria adhesion defects.

(A) Representative scanning electron microscopy images of hemocytes from the indicated genotypes of L3 wandering larvae after 30 minutes incubation with *S. aureus* live bacteria, at room temperature. Scale bar: 2 µm. **(B)** Representative transmission electron micrographs of

hemocytes from the indicated genotypes of L3 wandering larvae after 60 minutes incubation with *S. aureus* live bacteria, at room temperature. **(C)** Representative scanning electron microscopy images of hemocytes from the indicated genotypes of L3 wandering larvae after 30 minutes incubation with *E. coli* live bacteria, at room temperature. Scale bar: 1 µm. **(D)** Representative transmission electron micrographs of hemocytes from the indicated genotypes of L3 wandering larvae after 60 minutes incubation with *E. coli* live bacteria, at room temperature. Arrowheads and arrows indicate hemocyte membrane protrusions and internalized bacteria, respectively. **(E-F)** Hemocytes from the corresponding genotypes were incubated with *S. aureus* GFP (green) or *E. coli* GFP (green) live bacteria on ice (E), or with Cytochalasin D (F), for one hour (see Material and Methods section for further details). After fixation with 4% paraformaldehyde, hemocytes were stained with rhodamine phalloidin (red). Scale bar: 10 µm.

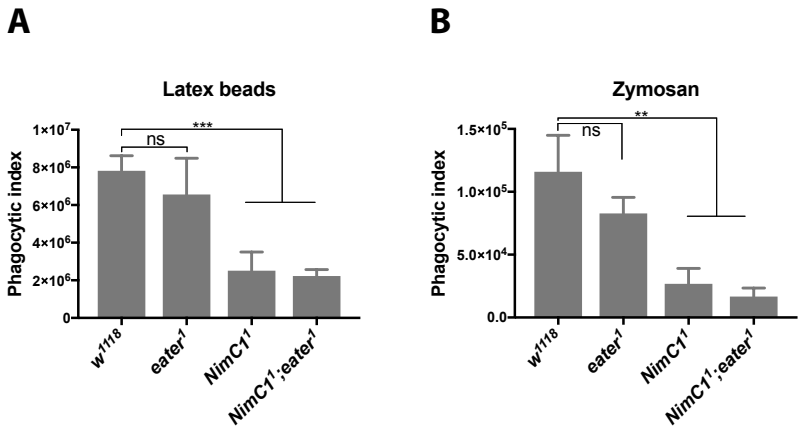

Figure 19. *NimC1[1]* hemocytes show impaired phagocytosis of latex beads and zymosan particles.

(A) Phagocytic index quantification of latex beads (Sigma-Aldrich) engulfment in hemocytes from *w[1118], eater[1], NimC1[1] and NimC1[1];eater[1]* L3 wandering larvae after 30 minutes incubation. Data are represented as mean ± SD from 5 independent experiments **(B)** Phagocytic index quantification of AlexaFluor™488 Zymosan (*S. cerevisiae*) BioParticles™ engulfment. Zymosan BioParticles were incubated with *w[1118]* or mutant hemocytes from L3 wandering larvae for 90 minutes. Data are represented as mean ± SD from 4 independent experiments. In (A) and (B), phagocytosis was quantified by flow cytometry, and the fluorescence of extracellular particles quenched by adding trypan blue. ** $p<0.01$, *** $p<0.001$, by Student t tests. ns: not significant.

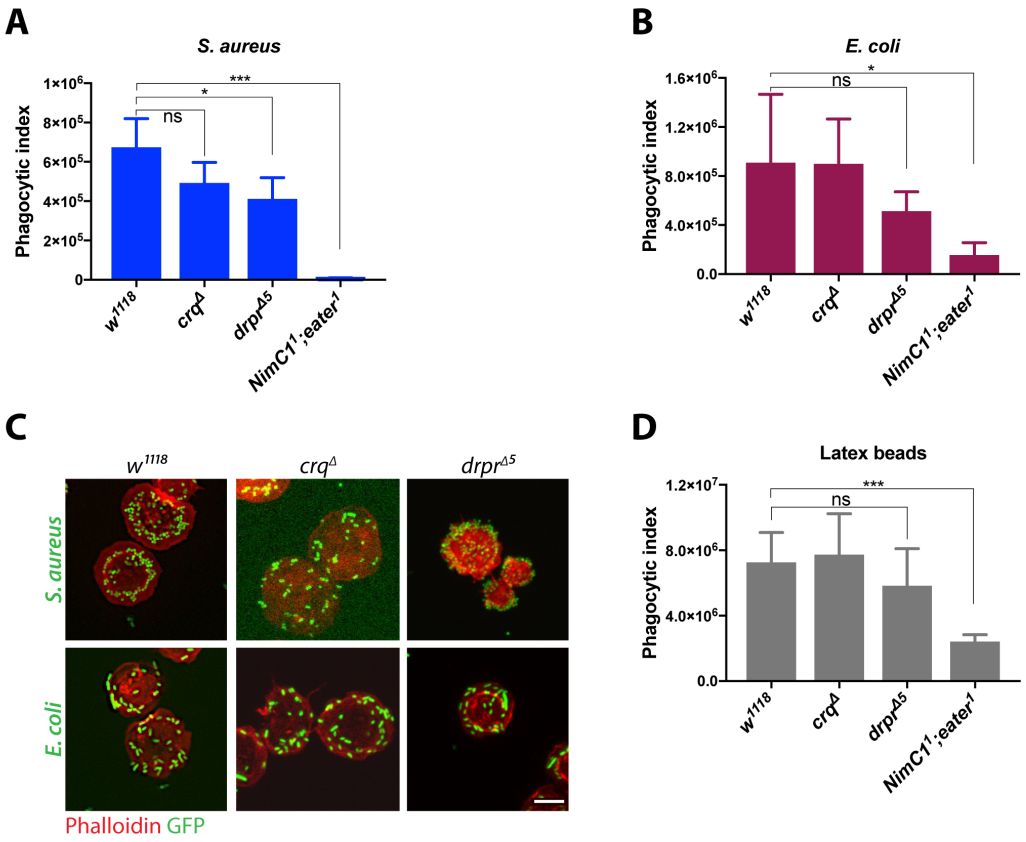

Figure 20. Phagocytosis in *draper* and *croquemort* mutants.

Phagocytosis of *S. aureus* **(A)** and *E. coli* **(B)** AlexaFluor™488 BioParticles™ (Invitrogen) in crq^{Δ} and $drpr^{\Delta 5}$ hemocytes mutants from L3 wandering larvae. $NimC1^{1};eater^{1}$ hemocytes were used as negative control. **(C)** crq^{Δ} and $drpr^{\Delta 5}$ hemocytes mutants show no binding defect of *S. aureus* (upper panel) and *E. coli* (bottom panel) bacteria. Hemocytes from the corresponding genotypes were incubated with live GFP bacteria (green) on ice for one hour (see Material and Methods section for further details). After fixation with 4% paraformaldehyde, hemocytes were stained with rhodamine phalloidin (red). Scale bar: 10 µm. **(D)** Phagocytosis of latex beads (Sigma-Aldrich) in crq^{Δ} and $drpr^{\Delta 5}$ hemocytes mutants from L3 wandering larvae. $NimC1^{1};eater^{1}$ hemocytes were used negative control. Data are represented as mean ± SD from 4 independent experiments * $p<0.05$, *** $p<0.001$, by Student t tests. ns: not significant.

Chapter 3: Characterization of Eater's role in hemocyte sessility in *Drosophila melanogaster*

Characterization of Eater's role in hemocyte sessility in *Drosophila melanogaster*

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[Manuscript in preparation, additional results will be added]

Abstract

Eater is a transmembrane receptor of the Nimrod family specifically expressed in mature *Drosophila* blood cells (hemocytes). Eater was initially identified for its crucial role in bacteria phagocytosis. However, in the last years, several works established a dual role of this protein as a key phagocytic receptor and a crucial adhesion molecule for hemocytes sessility in larval stages. In this study, we attempted to further characterize Eater functions in regards to hemocytes sessility and adhesion properties. We showed that, as in larval stages, Eater is required in adult hemocytes to confer adhesion properties and to enter the sessile state.

Moreover, *eater*^{1} adult flies have a decreased number of hemocytes, suggesting that blood cell sessility is an important requirement for their survival during adulthood. A preliminary functional analysis on possible downstream Eater targets led us to hypothesise that this receptor is most likely not regulating hemocyte sessility through transcriptional control of cell adhesion genes. In line with Eater's role as a tethering/adhesion molecule, Eater over-expression in hemocytes leads to the formation of enlarged cells compared to control. Bacteria phagocytosis, however, was decreased in this condition, suggesting that *eater* gene levels are critical for microbes' engulfment. We conclude that Eater serves as an important molecule providing initial contact and adhesion to the hemocyte target surface.

Introduction

Drosophila blood cells, or hemocytes, are important players of the cellular and humoral immune response, through the engulfment of pathogens, synthesis of antimicrobial peptides, and encapsulation of foreign objects (Melcarne et al., 2019; Vlisidou and Wood, 2015). *Drosophila* possesses three types of hemocytes, derived from a common precursor (or prohemocyte): plasmatocytes, crystal cells, and lamellocytes. Plasmatocytes, the most abundant cell type at all developmental stages, act as professional macrophages, mediating phagocytosis of bacteria and apoptotic cells (Gold and Brückner, 2015). In addition to these phagocytic tasks, plasmatocytes also contribute to tissue remodelling during metamorphosis and secretion of antimicrobial peptides in response to bacterial challenge (Martinek et al., 2008; Samakovlis et al., 1990). Crystal cells comprise the remaining part of the blood cell population and are responsible for melanin production and deposition at wound sites (Binggeli et al., 2014; Crozatier et al., 2004). Lamellocytes are large and adherent cells that are rarely present in unchallenged animals, but can differentiate in larval stages in response to specific stress signals, such as parasitation (Vlisidou and Wood, 2015). In this specific condition, lamellocytes mediate the encapsulation of the parasitoid eggs. *Drosophila* hematopoiesis occurs in two distinct spatiotemporal waves (Banerjee et al., 2019). A first, embryonic hematopoiesis gives rise to a defined number of plasmatocytes that will exponentially proliferate during larval stages, and to rare crystal cells (Holz, 2003; Tepass et al., 1994). At the larval stage, hemocytes are found in three compartments: (i) the lymph gland, that functions as a reservoir releasing hemocytes after parasitic infection, (ii) in circulation, and (iii) in the sessile patches (Crozatier and Meister, 2007; Evans et al., 2003; Honti et al., 2010; Jung, 2005; Lanot et al., 2001; Makhijani et al., 2011; Makhijani and Brückner, 2012). The second wave of hematopoiesis takes place in the lymph gland, where hemocytes progenitors differentiate during larval stages,

giving rise to mature plasmatocytes and crystal cells (Rugendorff et al., 1994). The lymph gland breaks at the onset of metamorphosis, releasing into the circulation the differentiated hemocytes. Sessile hemocytes are attached to the internal surface of the larval cuticule, forming patches, some of which are closely associated with secretory cells called oenocytes, as well as to the endings of peripheral neurons (Makhijani et al., 2011; Makki et al., 2014). Hemocytes continuously exchange between sessile patches and the circulation (Babcock et al., 2008; Welman et al., 2010). The network of peripheral neurons of the sessile patches controls hemocyte homing, adhesion, and proliferation through Activin-ß signalling (Makhijani et al., 2017). It has been shown, indeed, that in this sessile compartment hemocytes actively proliferate, establishing a larval hematopoietic tissue (Makhijani et al., 2017). During the pupal stage, this sessile population is disrupted, for then being newly rearranged during adulthood. Both embryonic and larval blood cells lineages persist into the adult fly, where they are also found in a sessile and circulating state. Examples of hemocytes clusters are found in the dorsal abdomen flanking the fly heart and close to the respiratory tract (Bosch et al., 2019). Recent studies have ruled out the presence of adult hemocyte proliferation (Bosch et al., 2019). In contrast, hemocyte immunosenescence is observed as blood cell number decays over time (Bosch et al., 2019; Horn et al., 2014; Lanot et al., 2001; Van De Bor et al., 2015).

A key hemocyte factor involved in hemocyte sessility is the receptor Eater, which is specifically expressed in mature hemocytes (Bretscher et al., 2015; Kocks et al., 2005). Eater is a transmembrane receptor of the Nimrod family that contains EGF-like repeats (Kocks et al., 2005; Kurucz et al., 2007). Eater has a dual role contributing to plasmatocyte phagocytosis and sessility. We have previously shown, indeed, that Eater is required cell-autonomously in plasmatocytes for hemocyte sessility (Bretscher et al., 2015). Moreover, in addition to their inability to enter the sessile state, hemocytes from *eater* null third instar larvae show spreading defects *ex vivo* (Bretscher et al., 2015). Nevertheless, Eater's role in sessility, and to a less extent in phagocytosis, has not been fully characterized during adulthood yet. Furthermore, it is still unknown whether Eater fulfils its functions by working as a cell adhesion receptor or as a signalling receptor, activating specific downstream pathways for cell adhesion and/or phagocytosis.

In the present work, we further investigated the role of Eater to decipher whether this protein also mediates blood cell sessility and adhesion during adulthood. We found that in $eater^{1}$ flies, hemocytes have adhesion defects and rarely form sessile clusters. Although present in the same number right after eclosion, hemocytes number declines significantly faster in $eater^{1}$ compared to *wild-type* flies. This suggests that hemocyte adhesion is critical for blood cell survival at the

adult stage. To address whether Eater engagement regulates the expression of genes involved in cell adhesion, we performed RNA-seq on naïve FACS-sorted *eater*^{1} and *wild-type* hemocytes from L3 wandering larvae. Analysis of differentially expressed genes identified some putative targets which are significantly downregulated in *eater*^{1} larval hemocytes with GO terms related to cell adhesion. However, the knock-down of those genes did not recapitulate the *eater* loss of sessility phenotype. Finally, we further characterized the role of Eater as an adhesion molecule by analysing the effects of its over-expression in hemocytes. In line with Eater's role in cell adhesion, Eater over-expressing hemocytes showed an enhanced cell spreading and lamellipodium formation. However, bacteria phagocytosis was decreased in those conditions, suggesting that *eater* expression levels need to be tightly regulated to perform efficient pathogen engulfment.

Results

Eater is required for hemocyte adhesion and sessility in adult flies

In the last years, several works have pointed out a dual role of Eater as a key receptor for *S. aureus* phagocytosis (Bretscher et al., 2015; Kocks et al., 2005; Melcarne et al., 2019) and a crucial molecule for hemocytes sessility in third instar larvae (Bretscher et al., 2015). We decided to characterize Eater functions in adult flies, to decipher whether this protein also plays a major role in blood cell sessility at this stage. We first started by investigating hemocytes adherence in regards to their ability to attach to the fly's integument. In *wild-type* animals, *srpmCherry.nls* tagged hemocytes were visible in circulation in the hemolymph, head, legs, as well as attached to the abdomen integument close to the fly heart, as described in (Bosch et al., 2019 (**Fig. 21A**). In *eater*^{1} mutant flies, hemocytes were detected in the legs, heads, thorax (probably in close proximity to the aorta), but rarely in the abdomen as a sessile population (**Fig. 21A**). This suggests that Eater is also required for hemocyte sessility during adulthood. No sessile hemocytes were also observed in dissected dorsal abdomens of *eater*^{1} flies at 5 days post-eclosion (**Fig. 21B**). To further characterize the adhesion properties of adult *eater*^{1} hemocytes we analysed their ability to attach to larval fat body cells that are found in emerging adults. The larval fat body undergoes a massive remodelling through tissue dissociation during metamorphosis, producing individual or clusters of large fat body cells in the pupa that persist in young flies. At the onset of adulthood, hemocytes are usually found attached to larval fat body cells (Nelliot et al., 2006)(**Fig. 21C**). Interestingly, *eater*^{1} hemocytes were not able to associate to these larval fat-cells, as a further indication of an altered cell adhesion (**Fig. 21C**). Of note, larval fat body cells were still eliminated with the same kinetics in *eater*^{1} and *wild-*

type flies, in agreement with a previous report showing that fat body remodelling in *Drosophila* is independent of hemocytes (Nelliot et al., 2006). We next analysed the morphological features of adult hemocytes upon *ex vivo* spreading. Surprisingly adult *wild-type* hemocytes are remarkably different from those of their third instar larval counterparts, notably in their spreading abilities **(Fig. 21D)**. The evident lamellipodium region observed in L3 wandering *wild-type* hemocytes was indeed absent in the adult blood cells. Moreover, hemocytes from *eater* flies were characterized by less and shorter filopodia protrusions compared to the larval ones.

Hemocytes with reduced sessility show decreased survival during adulthood

To further understand whether the altered hemocyte sessility in adult could have an impact on blood cell number, we quantified by flow cytometry the total number of *srpmCherry.nls* hemocytes from dissected *wild-type* and $eater^{1}$ flies at the following ages: 2, 5, 10 and 15 days (**Fig. 22A**). After eclosion, hemocyte number was similar in the two tested backgrounds. Starting from the first week of adult life, *wild-type* hemocytes begin a continuous decline in number over time (**Fig. 22A**). This is in agreement with several studies showing hemocytes immunosenescence in adult flies (Bosch et al., 2019; Horn et al., 2014; Lanot et al., 2001; Mackenzie et al., 2011; Van De Bor et al., 2015). Interestingly, this decline in hemocyte number was significantly faster in $eater^{1}$ flies, suggesting that blood cell sessility might be an important requirement for their survival during adulthood (**Fig. 22A**). Similar results were also obtained using adult flies containing the hemocyte marker *HmlΔdsred.nls* combined with the *eater* mutation (**Fig. 22B**).

To further understand the effects of sessility on adult hemocyte survival, we decided to analyse hemocytes behaviour in another background affecting hemocytes adhesion properties. It has been previously reported that over-expression of wild-type Rac1 in hemocytes disrupts the sessile hemocyte population (Zettervall et al., 2004)(**Fig. 22C**). Rac1 is a small GTPase of the Ras superfamily that has previously been involved in hemocyte migration in the *Drosophila* embryo (Paladi and Tepass, 2004; Stramer et al., 2005). Abdomen dissection of Rac1 over-expressing flies revealed the absence of sessile hemocytes attached to the dorsal integument. The same phenotype was observed upon *eater* silencing through *in vivo* RNAi (**Fig. 22D**). In addition, a remarkable decrease in hemocyte number was observed in adult Rac1 over-expressing flies compared to *wild-type* (**Fig. 22E**), but not in L3 wandering larvae (**Fig. 22F**), as a further indication that hemocytes sessility might contribute to their survival in adulthood.

RNA-seq of hemocytes from *eater^{1}* mutant third instar larvae

To better characterize the role of Eater as an adhesion receptor, we performed a transcriptomics analysis of *eater^{1}* versus *wild-type* unchallenged hemocytes. The key question we aimed to answer was whether Eater transcriptionally regulates cell adhesion genes. Therefore, to identify differentially expressed genes in *eater^{1}* compared to *wild-type* hemocytes, we collected hemolymph of naïve L3 wandering larvae harbouring the *HmlΔdsred.nls* fluorescent marker and isolated their hemocytes by FACS. We collected 20.000 to 30.000 cells from each pool of larvae, and all conditions were repeated in three different experiments. RNA was then extracted and RNA-seq was performed as described in the methods, resulting in an average of 53 million gene reads per treatment. We then performed a differential gene expression analysis, by looking at differences in gene expression between naïve *wild-type* and *eater^{1}* hemocytes (**Fig. 23A**). We found that 92 genes are to a significant extent differentially expressed between *eater^{1}* and *wild-type* hemocyte samples (**Fig. 23B)**, 59 of which being more transcribed, and 33 being significantly downregulated in *eater^{1}* hemocytes (Supplementary Table 1). Among the most up-regulated genes in *eater^{1}* we found several ribosomal RNA-encoding proteins, whereas different genes involved in cell adhesion were found among the down-regulated group. We decided to focus our attention on this latter category, and in particular on genes with GO terms related to cell adhesion and/or actin cytoskeleton remodelling machinery. The representative selected genes we chose are *spire (spir)*, *RhoGAP18B*, *Matrix metalloproteases1 (Mmp1)*, *Ecdysone-inducible gene L2 (ImpL2)*, *Activity-regulated cytoskeleton associated protein1 (Arc1)*. Gene expression validation through qRT-PCR did confirm a decreased level of transcription for *spir*, *Arc1*, and *ImpL2*, but not for *Mmp1* and *RhoGAP18B* in *eater^{1}* larval hemocytes (**Fig. 23C**). We then performed a functional characterization of *eater* modulated genes with the hope to discover factors that could modulate blood cell sessility. For this we analysed hemocyte sessility and adhesion in larvae expressing an RNAi against *spir*, *Arc1*, or *ImpL2*, under the *HmlΔGal4* driver. Hemocytes localization using *HmlΔGal4,UAS-GFP* did not reveal any significant defect in hemocyte sessility. Hemocytes were indeed still able to enter the sessile state, forming lateral and dorsal patches (**Fig. 23D**). In line with these observations, hemocyte spreading on a glass slide did not show impaired adherence *ex vivo* (**Fig. 23E**). It has previously been shown that *eater* null larvae harbour more hemocytes compared to control animal. We then also tested whether silencing of these selected genes could lead to an increase in hemocyte number, without affecting their sessility. However, hemocytes count from third instar larvae was *wild-type*-like in the tested silencing conditions (**Fig. 23F**). In conclusion, our functional analysis did not reveal any gene recapitulating the

eater loss of sessility phenotype.

To get a broader view of potential candidate genes, we relaxed our cutoff to a *p*-value < 0.05 instead of using an FDR of < 0.05 and performed GO Term analysis. Using these cutoffs, we found a significant enrichment of GO Terms of biological processes as shown in **Table 1**. Interestingly we found the processes "cell-cell adhesion" and "homophilic cell adhesion" enriched and the implicated genes are depicted in **Table 2**. While our functional analysis did not reveal any direct implication of some of those genes, future studies targeting the remaining genes or combining several of them might deliver fruitful results.

Eater expression levels control larval hemocyte adhesion properties

Next, we decided to further investigate the role of Eater as an adhesion molecule by analysing the effects of its over-expression in hemocytes. For this, *UAS-eater* transgenic flies were crossed to *HmlΔGal4* or *HmlΔGal4,UAS-GFP* driver flies. We first started by looking at hemocytes morphology and adhesion properties in L3 wandering larvae. Interestingly, phalloidin staining revealed that *eater* over-expression in larval hemocytes induced the formation of enlarged cells likely as a consequence of enhanced spreading (**Fig. 24A, B**). Moreover, image-based cytometry analysis of free-floating hemocytes revealed a similar cell size distribution in both control and *eater* over-expressing hemocytes (**Fig. 24C**). Enlarged hemocytes were also observed by *in vivo* imaging of the dorsal sessile patches (**Fig. 24D'''**). However, despite the presence of those enlarged and adherent cells, the segmental banding pattern observed all along the dorsal side of control larvae was partially disrupted in *eater* over-expressing condition (**Fig. 24D''**). Given this peculiar cell shape phenotype, we decided to analyse hemocytes spreading by over-expressing two other types of phagocytic receptors, Draper and Croquemort (Franc et al., 1996; Freeman et al., 2003). Both these proteins have been involved in the phagocytosis of *S. aureus* bacteria (as Eater) and apoptotic cells, and belongs to the Nimrod and CD36 family of proteins, respectively (Franc et al., 1996; Hashimoto et al., 2009; Kuraishi et al., 2007; Kurucz et al., 2007; Manaka et al., 2004; Tung et al., 2013). As shown in **Fig. 24E**, over-expression of either one of these receptors did not increase hemocytes spreading to the same extent as *eater* over-expression. Filopodia formation, however, appeared to be more enhanced in *draper* and *croquemort* over-expressing hemocytes. Those data would suggest a specificity of Eater, among other transmembrane receptors, in modulating cell shape when over-expressed. Finally, besides the enlarged and highly adherent hemocytes, phalloidin staining also revealed the presence of elongated flat cells, whose shape was reminiscent of lamellocytes (**Fig. 24F**, white arrow). Use of the lamellocyte marker

MSNF9MO-mCherry (*msn-mCherry*) revealed indeed the presence of individual and aggregated lamellocytes in *eater* over-expressing L3 wandering larvae (**Fig. 24G**). Finally, to further investigate the effects of *eater* over-expression in hemocytes, we counted the total hemocytes number (i.e. sessile and circulating) by flow cytometry in *HmlΔGal4,UAS-GFP* and *HmlΔGal4,UAS-GFP>UAS-eater* third instar larvae. Our analysis revealed that Eater, when over-expressed in hemocytes, promotes an increase in the total number of *Drosophila* blood cells. Around three times more hemocytes were indeed present compared to control larvae (**Fig. 24H**).

***eater* gene dosage plays a critical role in bacterial phagocytosis**

A specificity of Eater is its dual role in adhesion and phagocytosis (Bretscher et al., 2015; Kocks et al., 2005). We have shown that over-expression of *eater* increases adhesion, indicating that the level of this protein shapes adhesion properties. We next investigated whether *eater* over-expression in hemocytes could also enhance a stronger phagocytosis response. Therefore, we performed *ex vivo* phagocytosis assay using hemocytes from L3 wandering larvae. Surprisingly, *S. aureus* phagocytosis by hemocytes over-expressing *eater* was decreased compared to the control (**Fig. 25A**), although bacteria were still able to adhere to hemocyte surface (**Fig. 25B**). Interestingly, the phagocytic index of nonimmunogenic latex beads was wild type-like in *eater* over-expressing hemocytes (**Fig. 25C**), suggesting that *eater* expression has to be tightly regulated to perform efficient bacteria phagocytosis only. While examining *Hml>UAS-eater* hemocytes morphology in the absence of bacteria, we observed a fraction of the enlarged cells, previously described, presumably engulfing other larval blood cells, in a process reminiscent of the so-called cell cannibalism, or phagoptosis (**Fig. 25 D, E**). This striking phenotype could suggest that over-expressing *eater* specifically in hemocytes would result in a dysregulated hyper-phagocytic plasmatocyte engulfing healthy, but most importantly, non-invaders cells, while being less efficient to phagocyte bacteria.

Discussion

Eater has been initially identified as a transmembrane receptor involved in phagocytosis of bacteria and specifically expressed in mature plasmatocytes (Kocks et al., 2005). Recent studies have shed light on novel Eater functions in mediating plasmatocyte sessility and cell adhesion (Bretscher et al., 2015). In the present work we further characterized Eater requirement for hemocyte sessility in adult and larval blood cells by using either the previously

described *eater*^{1} mutant (Bretscher et al., 2015), or by generating novel transgenic animals containing the *eater* gene downstream of the *UAS* promoter, for over-expression studies.

We showed that Eater is required for hemocyte sessility in adult flies. Clusters of sessile plasmatocytes are indeed almost absent from the abdomen of *eater*^{1} animals. This could suggest that Eater ligand in the inner side of the cuticule is the same both at larval stages and adulthood. Nevertheless, we cannot exclude yet that Eater responds to different ligand(s) at different developmental stages. Furthermore, we showed that hemocytes from emerging flies need Eater in order to associate to larval fat cells, further indicating decreased adherent properties of adult *eater*^{1} hemocytes. Interestingly, adult hemocyte spreading on glass slide did not reveal any critical differences in the *ex vivo* cell spreading abilities between adult *wild-type* and *eater*^{1} hemocytes, with the former genotype showing already an evident decrease in the lamellipodium region compared to its larval counterpart. This altered morphology of *wild-type* adult hemocytes could reflect a distinct gene-expression pattern compared to the larval one, resulting in different adhesion abilities and/or functions.

In this study we uncovered a potential new role for cell adhesion in hemocyte survival during adulthood. Although present in the same number just after eclosion, hemocyte number declines significantly faster in *eater*^{1} compared to *wild-type* flies. We validated our hypothesis also by using another background affecting hemocyte sessility: over-expression of wild-type Rac1 (Zettervall et al., 2004). In this condition, we observed disruption of hemocyte sessility in third instar larvae, as well as in 5 days old flies. Interestingly, hemocyte number was significantly decreased in adult animals at 7 days post eclosion, suggesting once again that hemocyte adhesion is critical for blood cell survival in adult flies. We cannot exclude, however, that Rac1 and Eater work in the same pathway, therefore resulting in the same final phenotype. A more detailed analysis on other genetic backgrounds affecting hemocyte sessility will hence be required to fully understand this process. During larval development hemocytes of the sessile patches undergo massive proliferation, with EdU incorporation rates higher than the circulating one. These sessile hemocytes are in contact with the endings of peripheral neurons which establish a supportive niche by providing to the hemocytes Activin-ß, a trophic factor responsible for blood cell proliferation at these sites (Makhijani et al., 2017). During adulthood, hemocytes do not proliferate, but rather undergo an immunosenescence process (Bosch et al., 2019; Horn et al., 2014; Lanot et al., 2001; Mackenzie et al., 2011; Van De Bor et al., 2015). However, our observation that adult hemocytes need to be sessile in order to survive longer, led us to hypothesise that in correspondence to the adult sessile sites (e.g. in the abdomen and thorax), hemocytes are placed in a niche where they receive survival signals. It will be

interesting to identify the nature of the signal required for hemocyte survival and its mechanism of action. Would this trophic niche release the survival signal(s) in a continued manner during adulthood, or in a wave-type mode right after eclosion, therefore providing cues only for an initial and limited amount of time? Another possibility is that, with aging, the plasmatocyte decreases its adherent properties, thus detaching from the sessile clusters without the possibility to receive cues from the niche. Finally, we can also hypothesize that the niche requires hemocyte adhesion as a feedback to regulate its survival with the release of apposite signal(s). To address whether Eater regulates hemocytes sessility through transcriptional control of cell adhesion genes, we performed RNA-seq on unchallenged FACS-sorted *eater*^{1} and w^{1118} hemocytes from L3 wandering larvae. Unfortunately, our preliminary functional analysis on possible downstream Eater targets led us to hypothesize that this receptor might not be involved in cell adhesion genes regulation, even though we cannot definitively rule out this possibility yet. In our view, Eater would function as an adhesion molecule, only involved in "sticking" to target surfaces, providing an initial cell contact that would be required for the engagement of other receptor(s) involved in the stabilization of the complex and the subsequent triggering of intracellular signalling. In the absence of Eater, the plasmatocyte would not be able to provide this initial interaction with the target surface at all, with the consequent inability to correctly spread and entering the sessile state. Therefore, the downregulation of some genes involved in cell adhesion and/or actin cytoskeleton remodelling observed in our transcriptomic analysis might be an indirect consequence of a total lack of adherence in *eater*^{1} hemocytes. Given the inability of those cells to adhere, the entire intracellular machinery required for cell adhesion would constitutively be "turned off", resulting in a downregulation of genes with cell adhesion. In agreement with Eater's role as a "sticky" adhesion molecule, over-expression of this receptor in larval plasmatocytes resulted in cells with enhanced cell spreading and lamellipodium formation. Moreover, this effect appeared to be specific for Eater only, since over-expression of either *croquemort* or *draper* (other transmembrane receptors belonging to the CD36 or Nimrod family, respectively) did not affect hemocyte spreading to the same extent as Eater. Therefore, in larval stages hemocyte adhesion is controlled by *eater* in a dose-dependent way. It will be worth investigating in future studies the migration capacity of those enlarged cells, to analyse whether their enlarged lamellipodium area would allow them to migrate faster. Furthermore, *S. aureus* phagocytosis was impaired in hemocytes over-expressing *eater*, showing that *eater* expression level does not influence hemocyte adhesion only, but also microbe engulfment. Prior to receptor engagement, and all along particle internalization, phagocytic receptors undergo lateral mobility in the phagocyte membrane. This lateral

clustering of receptors is a crucial step for efficient phagocytosis (Freeman and Grinstein, 2014). We could assume that having too many Eater proteins at the plasmatocyte surface would (still) allow the binding to its cognate microbe target, but might prevent the required and proper clustering of receptors and/or co-receptors in correspondence of the phagocytic cup. On the other end, a saturation of the phagocytic machinery could also come from the intracellular side. What could happen is that the engagement, and consequent activation, of too many phagocytic receptors might not correctly activate all the downstream signalling events required for complete phagocytosis. Therefore, the particle might be engulfed, but blocked in the early phagosome maturation process. Surprisingly, despite this microbe engulfment defect, *eater* over-expression lead to hyper-activated macrophages engulfing, most probably, neighbouring blood cells. This is in line with a previous study showing that cells with enhanced phagocytic activity would induce engulfment and apoptosis of neighbouring wild-type cells (Li and Baker, 2007). It will be interesting to assess whether also in this condition the engulfed cells would undergo phagocytosis-dependent cell death (or phagoptosis), even though our flow cytometry counting experiments revealed an even higher hemocyte count in *Hml>UAS-eater* larvae, suggesting that maybe those cells are more proliferative. Interestingly, our data also suggest that *eater* over-expression might drive the expression of genes inducing lamellocytes differentiation. Lamellocytes are a type of hemocyte which is generally not found in healthy larvae. However, upon specific stress conditions they can increase very much in number in the hemolymph by differentiation from their direct lineage precursor, or by transdifferentiation of the sessile plasmatocytes (Anderl et al., 2016; Honti et al., 2010; Stofanko et al., 2010; Vlisidou and Wood, 2015). Preliminary data indicate that the lamellocytes present in Eater-overexpressing larvae are a mix of those two differentiating conditions (data not shown). It will be interesting, in future works, to perform encapsulation experiments in this genetic background to analyse how those hemocytes (both lamellocytes and enlarged plasmatocytes) adhere to the parasitoid egg, in particular whether they are more adherent and whether they are functionally more efficient in resolving the parasitoid infection.

To conclude, this work provided new important clarifications on the Nimrod receptor Eater in regards to its ability in mediating hemocyte sessility. Despite a role of Eater as signalling receptor cannot be entirely ruled out yet, we believe that this protein functions as an adhesion molecule, anchoring the hemocyte to its ECM substratum.

Material and Methods

Drosophila stocks and methodology

Drosophila stocks were reared at 25°C on standard fly medium consisting of 6% cornmeal, 6% yeast, 0.62% agar, 0.1% fruit juice (consisting on 50% grape juice and 50% multifruits+multivitamin juice), supplemented with 10.6 g/L moldex and 4.9 ml/L propionic acid. Third instar (L3) wandering larvae were selected 110-120 hours AEL. *Wild-type* w^{1118} (BL5905) flies were used as controls, unless indicated otherwise. The following transgenic lines and mutants were used: $eater^{1}$ (Bretscher et al., 2015), *HmlΔdsred.nls* (Bretscher et al., 2015), $eater^{1}$*HmlΔdsred.nls* (Melcarne et al., 2019), *HmlΔGal4,UAS-GFP* (Sinenko and Mathey-Prevot, 2004), *HmlΔGal4* (Sinenko and Mathey-Prevot, 2004), *UAS-Eater-IR* (VDRC 30097), *UAS-eater* (this study), *srpmCherry.nls* (Gyoergy et al., 2018), $eater^{1}$*srpmCherry.nls* (this study), *UAS-Rac1* (Zettervall et al., 2004), *msn-mCherry* (Tokusumi et al., 2009), *UAS-spire-IR* (Bloomighton 30516), *UAS-Arc1-IR* (Bloomighton 25954), *UAS-ImpL2-IR* (Bloomighton 55855), *UAS-Mmp1-IR* (Bloomighton 31489), *UAS-RhoGAP18B-IR* (Bloomighton 31165), *UAS-draper* (Courtesy from E. Kurant), *UAS-croquemort* (Courtesy from E. Kurant).

Hemocyte phalloidin staining

Extracted hemocytes from L3 wandering larva or adult flies were allowed to adhere on a glass slide for 30 minutes and subsequently fixed in 4% paraformaldehyde PBS. Cells were then stained with diluted 1/100 AlexaFluor488- or rhodamine-phalloidin (Molecular Probes™) for two hours, and after with a 1/20000 dilution of 4',6- diamidino-2-phenylindole DAPI (Sigma). Samples were mounted in anti-fading agent Citifluor AF1 (Citifluor Ltd.) and imaged with an Axioplot Imager.Z1 Zeiss coupled to an AxioCam MRm camera (Zeiss).

Hemocyte quantification by flow cytometry

Hemocyte quantification by release from adult flies was performed as follows. For each time point and genotype, 5 females containing the *srpmCherry.nls* hemocyte marker were dissected in 240 µL of Schneider's insect medium (Sigma-Aldrich) containing 1 nM phenylthiourea (PTU, Sigma). First, the head was removed with forceps and carefully pinched to release the hemocyte content. Next, the thorax was crashed and squeezed multiple times in order to release the attached hemocytes. The abdomen was subsequently open ventrally and pocked in the dorsal side in order to detach the sessile hemocyte population. The remaining carcass was further crashed in order to release the remaining hemocytes from the rest of the fly integument, including the leg's one. The obtained hemocyte suspension was then filtered and 60 µL were analysed by flow cytometry.

For larval hemocyte counting, 5 L3 wandering third instar larvae per genotype expressing the *HmlΔGal4,UAS-GFP* hemocyte marker were bled into 150 µL of Schneider's insect medium (Sigma-Aldrich) containing 1 nM phenylthiourea (PTU, Sigma). Larvae were vortexed for one minute in 1X PBS to allow the detachment of sessile hemocytes (Petraki et al., 2015). 75 µL of the obtained hemocyte suspension was analysed by flow cytometry.

A BD Accuri C6 flow cytometer was used to analyse hemocyte numbers. Hemocytes were first selected from debris by plotting FSC-A against SSC-A on a logarithmic scale in a dot plot. Cells were then gated for singlets by plotting FSC-H versus FSC-A. Untagged and *HmlΔGal4,UAS-GFP* or *srpmCherry.nls* animals were used to define the gates for hemocyte population, using a FL1 and FL2 detector, respectively.

Hemocyte size measurement of free-floating cells

Third instar larvae of the corresponding genotype were bled in 1X PBS without calcium and magnesium, supplemented with EDTA 5mM. Invitrogen™ Tali™ Image-based Cytometer machine was used to measure hemocytes size in suspension of more than 7 000 cells per genotype.

Live imaging of L3 wandering third instar larvae

For whole larval imaging, cleaned larvae were placed on a pre-chilled glass support and imaged with a Leica DFC300FX camera and Leica Application Suite.

For live imaging of dorsal sessile patches, larvae were mounted on a double side tape on a glass slide, with their dorsal side up. A cover glass was then placed dorsally and taped to the glass slide. Images of sessile patches were captured with Axioplot Imager.Z1 Zeiss coupled to an AxioCam MRm camera (Zeiss) with a 10x objective.

***Ex vivo* phagocytosis assays**

(1) *Ex vivo* phagocytosis assay of *S. aureus* bacteria was performed using *S. aureus* AlexaFluor™488 BioParticles™ (Invitrogen), following manufacturer's instructions. Briefly, hemocytes from L3 wandering larvae were bled into 150 µL of Schneider's insect medium (Sigma-Aldrich) containing 1 µM phenylthiourea (PTU, Sigma-Aldrich) to block melanization. The obtained hemocyte suspension was then placed in a 1.5 mL low binding tube (Eppendorf) and 2 x 10^7 AlexaFluor™488 BioParticles™ were added. After an incubation of 60 minutes the samples were placed on ice in order to stop the phagocytosis reaction. The fluorescence of extracellular particles was quenched by adding 0.4% trypan blue (Sigma-

Aldrich) diluted 1/3. Phagocytosis was quantified using a flow cytometer (BD Accuri C6, USA) by measuring the fraction of cells phagocytosing, and their fluorescent intensity. The following formula were used to calculate the phagocytic index:

$$\textbf{Fraction of hemocytes phagocytosing (f)} = \frac{[number\ of\ hemocytes\ in\ fluorescence\ positive\ gate]}{[total\ number\ of\ hemocytes]}$$

$$\textbf{Phagocytic index (PI)} = [Mean\ fluorescence\ intensity\ of\ hemocytes\ in\ fluorescence\ positive\ gate]\ x\ f$$

(2) Phagocytosis of green fluorescent latex beads (1 µm diameter, Sigma-Aldrich) was performed following the same technical procedure described in (1). 0.2 µg of beads were added to each sample.

RNA extraction, RNA sequencing and analysis

Hemocytes were collected by dissecting L3 larvae in 120 µl PBS 1X droplet on glass slide. In order to specifically isolate hemocytes from other unwanted cells of the hemolymph, we used larvae expressing the *HmlΔdsred.nls* fluorescent marker, which is specifically expressed in plasmatocytes and crystal cells (Goto et al., 2003; Leitão and Sucena, 2015). The extracted hemolymph was subjected to fluorescence activated cell sorting (FACS) to isolate plasmatocyte/crystal cell populations. Flow cytometer scatter-plot outputs were analysed to delineate hemocyte population based on nucleic red-fluorescent signal. Cells were then resuspended in TRIzol and total RNA was extracted with phenol-chloroform RNA extraction technique. A Fragment Analyzer (Agilent Technologies, Inc., Santa Clara, CA 95051, USA) was used to assess RNA quality of the obtained samples. RNA-seq libraries were prepared using 73-100 ng of total RNA and the Illumina TruSeq Stranded mRNA reagents (Illumina; San Diego, California, USA) according to the supplier's instructions. The bcl2fastq Conversion Software (v. 2.20, Illumina; San Diego, California, USA) was used to demultiplex sequencing data. The quality of the resulting reads was assessed with ShortRead (v. 1.28.0). Reads were then aligned to the reference genome (*Drosophila_melanogaster* BDGP6 dna.toplevel.fa) with TopHat (v2.1.0) and Bowtie (2.2.6.0). Mapping over exon-exon junctions was permitted by supplementing annotations (Drosophila_melanogaster BDGP6.87 GTF). Reads were counted with the featureCounts function (Rsubread 1.24.2). Differential expression analysis was performed with edgeR (3.26.4) and limma (3.40.2). Gene Ontology (GO) analysis was performed with Gorilla (online version http://cbl-gorilla.cs.technion.ac.il/ , July and August 2019). Two unranked lists of genes were compared, where the background set of genes was all genes with expressed with minimum of 10 CPM reads from all three combined unchallenged

hemocyte reads. The target set of genes was determined by the results of the differential expression analysis of the respective treatment with the following cutoffs: CPM > 5, P-value < 0.05, FC > +/-1.88.

For gene expression validation, RNA was extracted from the selected genotypes using TRIzol reagent (Invitrogen). NanoDrop ND-1000 spectrophotometer was used to assess RNA quality and quantity and 500 ng of total RNA was used to generate cDNA using SuperScript II (Invitrogen, Carlsbad, California, United States). Finally, qRT-PCR was performed using the PowerUP Master Mix (Roche). Expression values were normalized to *RpL32.*

Statistical analysis

Experiments were repeated at least three times independently and values are represented as the mean ± standard deviation. Data were analysed using GraphPad Prism 7.0. *p*-values were determined with Student t-tests, unless indicated otherwise. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ns: not significant.

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Authors' contribution

CL, SR, and BL designed the study. CM performed the experiments. JD and ER performed RNA seq experiments and the bioinformatic analyses. MP generated the *UAS-eater* construct. CM and BL analysed the data and wrote the manuscript.

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Figures

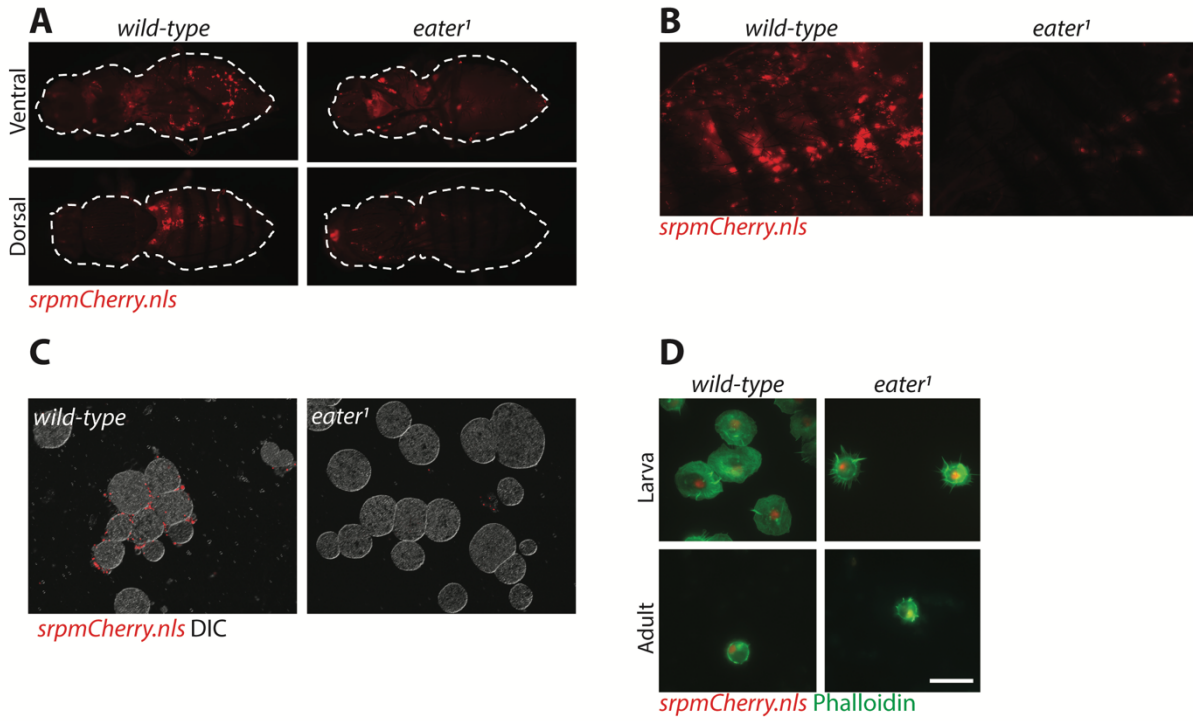

Figure 21. Eater is required for hemocyte adhesion and sessility in adult flies.

(A) Ventral and dorsal view of adult *wild-type* and *eater*^{1} *Drosophila srpmCherry.nls* at day 10 post eclosure. The fly boundaries are delimited by a white dashed line **(B)** Dissected abdomens of adult *wild-type* and *eater*^{1} *srpmCherry.nls* at day 5 post eclosure. Abdomen of the corresponding genotype was dissected in Schneider's insect medium (Sigma-Aldrich) and fixed in 4% paraformaldehyde PBS before mounting. **(C)** Larval fat body cells (grey) from dissected *wild-type* and *eater*^{1} young adult flies expressing the *srpmCherry.nls* hemocyte marker (red). **(D)** Representative images of fixed w^{1118} and *eater*^{1} *srpmCherry.nls* (red) hemocytes from the indicated developmental stages. Hemocytes were extracted by larval bleeding or adult dissection, allowed to spread for 30 minutes on glass slide, and stained with AlexaFluor™488 phalloidin (green). Scale bar: 20µm.

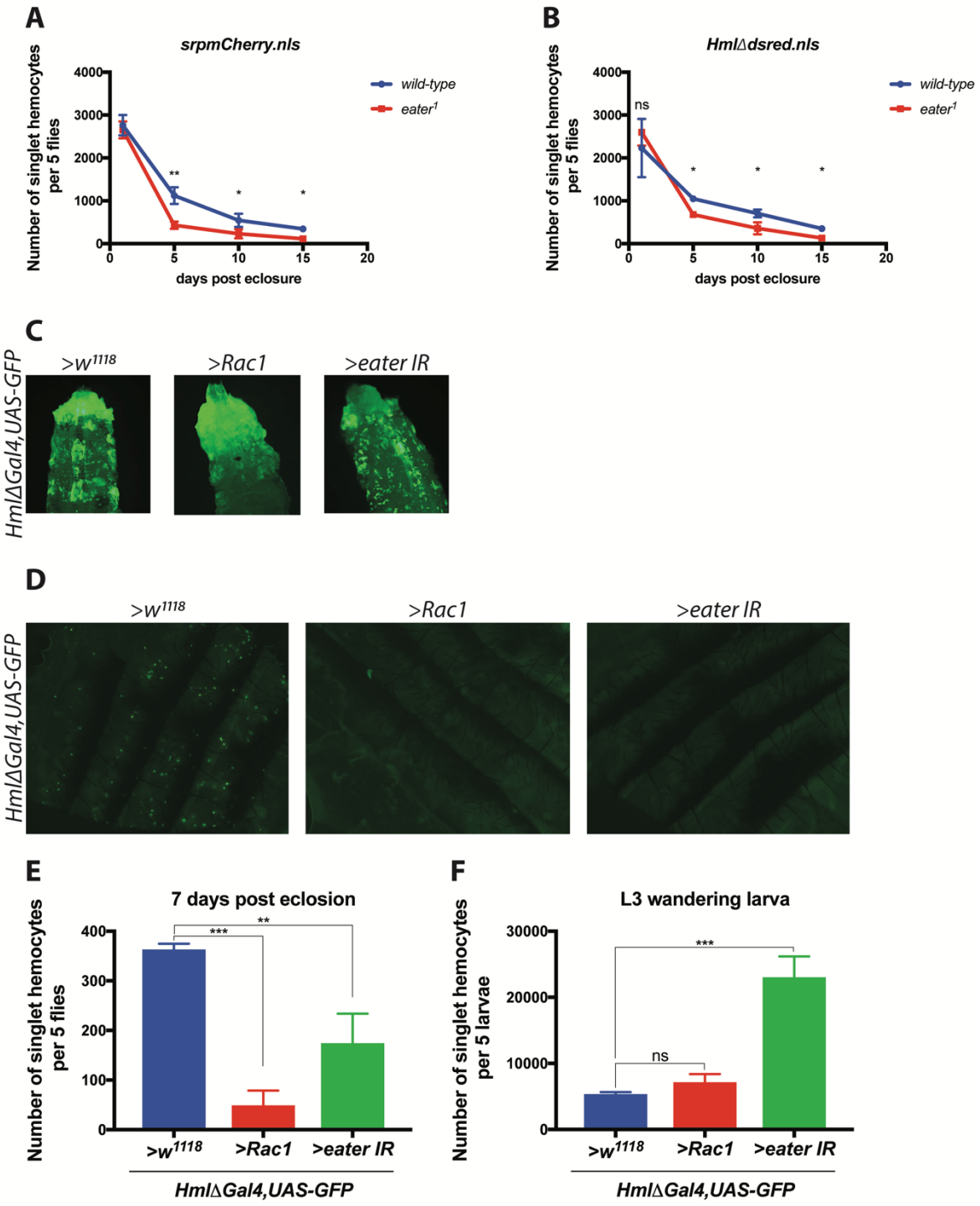

Figure 22. Hemocytes with reduced sessility show decreased survival during adulthood. **(A-B)** Total hemocyte count per 5 animals at the indicated time points from w^{1118} and *eater*^{1} *srpmCherry.nls* (A) and *HmlΔdsred.nls* flies (B). **(C)** Dorsal view of the posterior-most abdominal segments of third instar larva of the indicated genotypes, expressing *UAS-GFP* in plasmatocytes driven by *HmlΔ-GAL4*. **(D)** Dissected abdomens of control, Rac1 over-expressing, and *eater* RNAi expressing *HmlΔ-GAL4,UAS-GFP* adult flies at day 5 post eclosure. The abdomens were dissected in Schneider's insect medium (Sigma-Aldrich) and

fixed in 4% paraformaldehyde PBS before mounting on a glass slide. **(E)** Total hemocyte count per 5 animals at the indicated time point from *HmlΔ-GAL4,UAS-GFP* adult flies. **(F)** Number of singlet peripheral hemocytes from L3 wandering *HmlΔ-GAL4,UAS-GFP* larvae of the indicated genotypes. Results in (E) and (F) are represented as a sum of 5 animals. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, by Student t tests. ns: not significant.

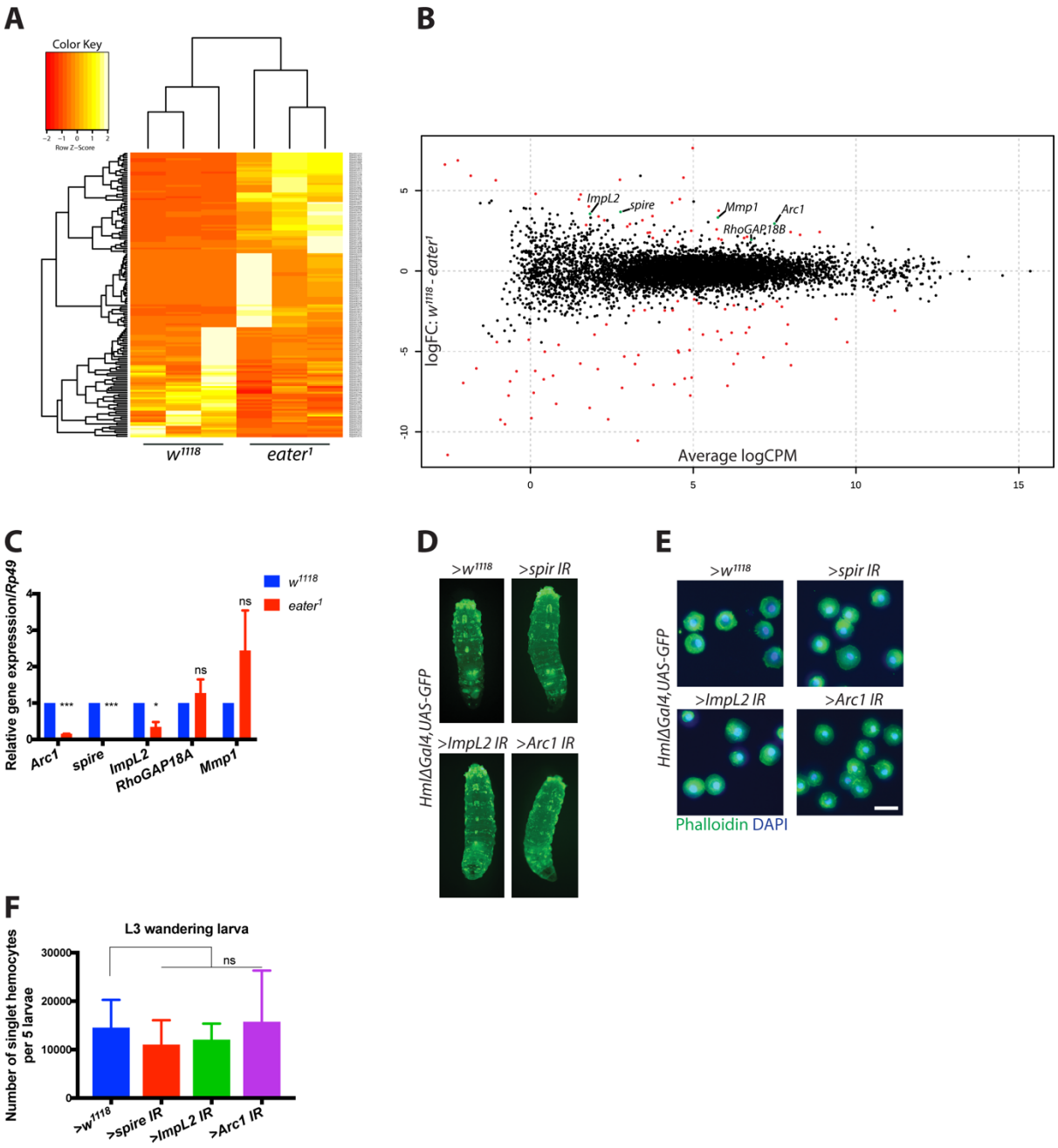

Figure 23. RNA-seq of hemocytes from *eater*^{1} mutant third instar larvae.

(A) Heat map of RNA-seq expression data displaying the top 150 differentially regulated genes between *wild-type* and *eater*^{1} hemocytes, sorted by p-value. Each column represents the value for one replicate experiment, whereas the colours indicate the normalized z-score in the corresponding experiment. **(B)** Mean-difference plot of the top 5000 differentially expressed genes is w^{1118} versus *eater*^{1} hemocytes from naïve third instar larvae. Genes with FDR < 0.05 are labelled red, and among those, the selected candidate genes *ImpL2*, *spire*, *Mmp1*, *RhoGAP18B*, and *Arc1* are specifically labelled green. Positive y-values indicate gene down-regulation in *eater*^{1}, whereas negative y-values gene up-regulation compared to *wild-type*. **(C)**

Gene expression validation by qRT-PCR of the selected genes in *eater*^{1} third instar larvae hemocytes. **(D)** Whole larva images of L3 wandering larvae of the indicated genotypes expressing *UAS-GFP* in plasmatocytes driven by *HmlΔ-GAL4*. **(E)** Representative images for fixed *HmlΔ-GAL4,UAS-GFP* hemocytes from the indicated genotypes of L3 wandering larvae. Hemocytes were extracted by larval bleeding, allowed to spread for 30 minutes on glass slide, and stained with AlexaFluor™488 phalloidin (green). Cell nuclei are shown in DAPI (blue). Scale bar: 20µm. **(F)** Number of singlet peripheral hemocytes per 5 L3 wandering *HmlΔ-GAL4,UAS-GFP* larvae of the indicated genotypes. * $p<0.05$, *** $p<0.001$, by Student t tests. ns: not significant.

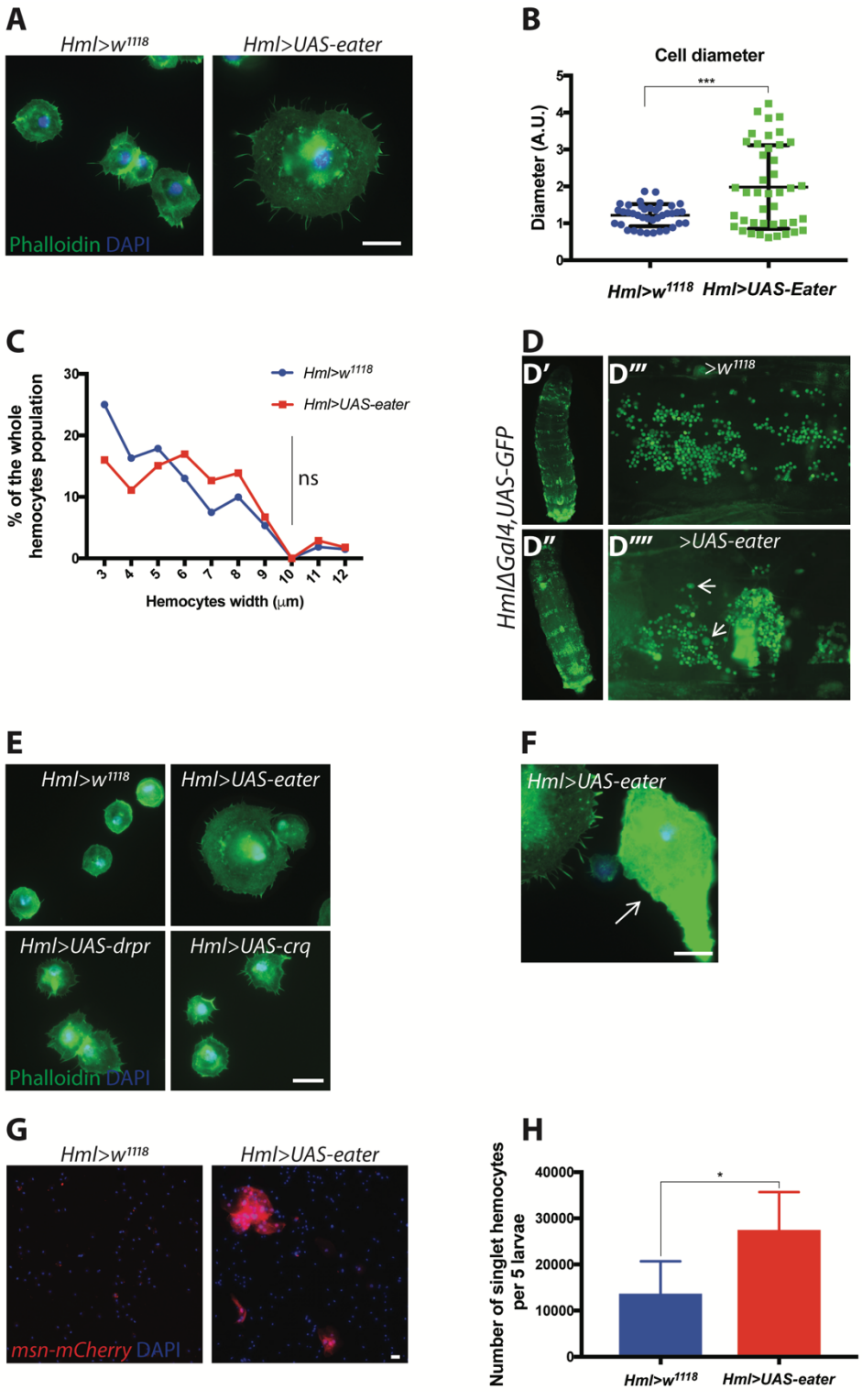

Figure 24. *eater* levels control larval hemocyte adhesion properties.

(A) Representative images of fixed hemocytes from the indicated genotypes. Hemocytes were extracted by larval bleeding, allowed to spread for 30 minutes on glass slide, and stained with AlexaFluor™488 phalloidin (green). Scale bar: 20µm. **(B)** Plasmatocytes diameter

determination of the indicated genotype by the Fiji software. The diameter for more than 30 cells per sample is shown. **(C)** Size distribution of free floating hemocytes from L3 wandering larvae of the indicated genotypes. Hemocyte size was measured with TALI imaged-based cytometer directly after larval bleeding of more than 7 000 cells per sample. ns: not significant by Kolmogorov-Smirnov test. **(D)** Whole larva images of *wild-type* and *eater* over-expressing third instar larvae. The dorsal side of the animal is shown (D' and D''). D''' and D'''' show live imaging of dorsal sessile patches of the corresponding genotype. **(E,F)** AlexaFluor™488 Phalloidin staining of fixed hemocytes (green) of the indicated genotypes. Cell nuclei are shown in DAPI (blue). White arrow in (F) indicate a lamellocyte-like cell in Eater over-expressing background. Scale bar: 20µm. **(G)** Representative images of fixed control or Eater over-expressing hemocytes expressing the lamellocyte marker *msn-mCherry* (red). Cell nuclei are shown in DAPI (blue). Scale bar: 20µm. **(H)** Number of singlet peripheral hemocytes per 5 L3 wandering *HmlΔ-GAL4,UAS-GFP* and *HmlΔ-GAL4,UAS-GFP >UAS-eater* larvae. * $p<0.05$, *** $p<0.001$, by Student t tests. ns: not significant.

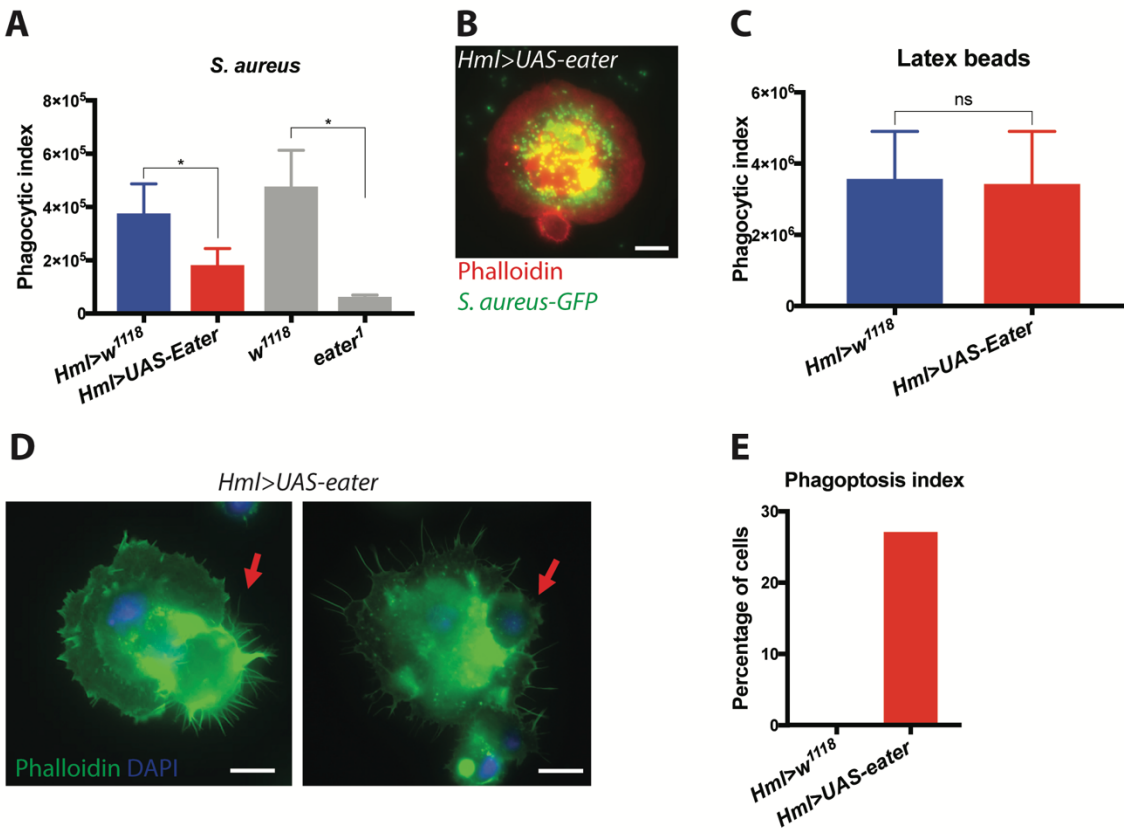

Figure 25. *eater* gene dosage plays a critical role in bacterial phagocytosis.

(A) Ex vivo phagocytosis assay using *S. aureus* AlexaFluor™488 BioParticles™ (Invitrogen). Hemocytes from third instar wandering larvae were incubated with *S. aureus* AlexaFluor™488 BioParticles™ (Invitrogen) for 1 hour at room temperature. The phagocytic index was calculated as described in the Material and Methods section. **(B)** Representative image of a plasmatocyte from an *eater* over-expressing larva incubated with *S. aureus*-GFP bacteria. After incubation, the sample was fixed with 4% paraformaldehyde and hemocytes were stained with rhodamine phalloidin (red). **(C)** Phagocytic index quantification of green latex beads (Sigma-Aldrich) engulfment in hemocytes from the indicated genotypes of L3 wandering larvae after 30 minutes incubation. **(D)** Representative images of phagoptosis events (red arrow) in fixed *eater* over-expressing hemocytes from L3 wandering larvae. Hemocytes were stained with AlexaFluor™488 phalloidin (green). Cell nuclei are shown in DAPI (blue). **(E)** Visual quantification of phagoptosis events in control and Eater over-expressing hemocytes. More that 100 cells per genotype were observed. In (A-E) *HmlΔ-GAL4* was used to as a hemocyte specific driver. * $p<0.05$, by Student t tests. ns: not significant.

Tables

GO Term	Description	P-value	FDR q-value
GO:0098742	Cell-cell adhesion via plasma-membrane adhesion molecules	5.1E-5	3.43E-1
GO:0007156	Homophilic cell adhesion via plasma-membrane adhesion molecules	2.17E-4	7.29E-1
GO:0045053	Protein retention in Golgi apparatus	2.73E-4	6.11E-1
GO:0097435	Supramolecular fiber organization	3.33E-4	5.6E-1
GO:0051085	Chaperone cofactor-dependent protein refolding	9.41E-4	1E0
GO:51084	"de novo" post-translational protein folding	9.41E-4	1E0

Table 1. Gene Ontology Term analysis

The table shows a GO term enrichment of biological processes in the genes significantly downregulated in *eater*^{1} hemocytes from our transcriptomic analysis. We found 6 significant GO terms enriched. Interestingly, cell adhesion terms are enriched in this condition (see "Cell-cell adhesion via plasma-membrane adhesion molecules" and "Homophilic cell adhesion via plasma-membrane adhesion molecules").

GO Term description	Genes
Cell-cell adhesion via plasma-membrane adhesion molecules	*ecdysone-inducible gene L2, cadherin 87a, gliolectin, lachesin, scab, echinoid, kugelei*
Homophilic cell adhesion via plasma-membrane adhesion molecules	*ecdysone-inducible gene L2, cadherin 87a, lachesin, echinoid, kugelei*

Table 2.

List of genes belonging to the indicated GO terms from Table 1.

CG	Full name	Gene	FBgn	FC	logFC	logCPM	Pvalue	FDR
CG33114	Guanylyl cyclase at 32E	Gyc32E	FBgn0010197	8998.38	-13.14	4.90	0.00	0.00
-	-	CR40639	FBgn0085758	1460.56	-10.51	7.57	0.00	0.00
CG2759	white	w	FBgn0003996	506.70	-8.98	6.00	0.00	0.00
CG42584	Ionotropic receptor 76a	Ir76a	FBgn0260874	471.42	-8.88	3.01	0.00	0.00
CG8166	unc-5	unc-5	FBgn0034013	409.84	-8.68	3.62	0.00	0.00
CG17839	-	CG17839	FBgn0036454	392.07	-8.61	2.73	0.00	0.00
-	-	CR40641	FBgn0085760	325.51	-8.35	5.08	0.00	0.00
CR45860	28S ribosomal RNA pseudogene:CR45860	28SrRNA-Psi:CR45860	FBgn0267520	318.21	-8.31	3.94	0.00	0.00
-	-	CR40766	FBgn0085773	213.79	-7.74	7.79	0.00	0.00
-	-	CR40679	FBgn0085766	167.31	-7.39	2.92	0.00	0.00
CR45855	28S ribosomal RNA pseudogene:CR45855	28SrRNA-Psi:CR45855	FBgn0267515	149.37	-7.22	5.46	0.00	0.00
CR45859	28S ribosomal RNA pseudogene:CR45859	28SrRNA-Psi:CR45859	FBgn0267519	140.44	-7.13	8.63	0.00	0.00
CR45845	ribosomal RNA primary transcript:CR45845	pre-rRNA:CR45845	FBgn0267505	137.30	-7.10	6.33	0.00	0.00
CR40741	28S ribosomal RNA pseudogene:CR40741	28SrRNA-Psi:CR40741	FBgn0085771	130.14	-7.02	6.95	0.00	0.00
-	-	CR41583	FBgn0085805	102.87	-6.68	7.10	0.00	0.00
-	-	CR41607	FBgn0085817	98.29	-6.62	7.28	0.00	0.00
-	-	CR41613	FBgn0085822	91.57	-6.52	2.82	0.00	0.00
-	-	CR41621	FBgn0085827	88.32	-6.46	2.99	0.00	0.00
CR45848	28S ribosomal RNA pseudogene:CR45848	28SrRNA-Psi:CR45848	FBgn0267508	67.12	-6.07	4.15	0.00	0.00
CR34096	mitochondrial small ribosomal RNA	mt:srRNA	FBgn0013688	57.98	-5.86	9.96	0.00	0.00
CR41609	28S ribosomal RNA pseudogene:CR41609	28SrRNA-Psi:CR41609	FBgn0085819	54.96	-5.78	5.14	0.00	0.00
CR41602	18S ribosomal RNA pseudogene:CR41602	18SrRNA-Psi:CR41602	FBgn0085813	54.81	-5.78	8.15	0.00	0.00
CG11697	Receptor accessory protein like 1	Reepl1	FBgn0030313	52.65	-5.72	3.46	0.00	0.00
-	-	CR41544	FBgn0085799	41.35	-5.37	8.88	0.00	0.00
-	-	CR42195	FBgn0085828	38.99	-5.28	4.58	0.00	0.00
-	-	CR41535	FBgn0085795	37.72	-5.24	4.97	0.00	0.00
CR45851	28S ribosomal RNA pseudogene:CR45851	28SrRNA-Psi:CR45851	FBgn0267511	35.24	-5.14	7.98	0.00	0.00
-	-	CR41619	FBgn0085825	33.45	-5.06	6.13	0.00	0.00
-	-	CR40668	FBgn0085764	30.93	-4.95	6.06	0.00	0.00
-	-	CR40642	FBgn0085761	30.27	-4.92	6.40	0.00	0.00
CR40596	28S ribosomal RNA pseudogene:CR40596	28SrRNA-Psi:CR40596	FBgn0085753	23.01	-4.52	9.19	0.00	0.00
CG42768	Muscle-specific protein 300 kDa	Msp300	FBgn0261836	21.47	-4.42	11.01	0.00	0.00
-	-	CR40712	FBgn0085768	19.30	-4.27	7.07	0.00	0.00
CG4120	Cyp12c1	Cyp12c1	FBgn0036806	15.47	-3.95	6.37	0.00	0.00
CG10157	Gamma-interferon-inducible lysosomal thiol reductase 2	GILT2	FBgn0039099	15.40	-3.94	3.07	0.00	0.02
CG34076	mitochondrial NADH-ubiquinone oxidoreductase chain 3	mt:ND3	FBgn0013681	14.32	-3.84	7.64	0.00	0.00
-	-	CR40959	FBgn0085777	14.27	-3.84	7.20	0.00	0.00
-	-	CR41590	FBgn0085807	13.43	-3.75	6.73	0.00	0.01
CG31918	Neprilysin-like 3	Nepl3	FBgn0031678	12.33	-3.62	5.82	0.00	0.00
-	-	CR40640	FBgn0085759	11.33	-3.50	7.59	0.00	0.01
CR31400	Heat shock RNA omega	lncRNA:Hsromega	FBgn0001234	10.04	-3.33	8.71	0.00	0.01
CG15093	-	CG15093	FBgn0034390	9.87	-3.30	3.16	0.00	0.00
CG31075	-	CG31075	FBgn0051075	7.93	-2.99	10.05	0.00	0.00
CG7002	Hemolectin	Hml	FBgn0029167	5.53	-2.47	11.67	0.00	0.02
CG18661	-	CG18661	FBgn0040964	5.48	-2.45	3.66	0.00	0.01
CG13397	-	CG13397	FBgn0014417	5.47	-2.45	4.60	0.00	0.00
CG34086	mitochondrial NADH-ubiquinone oxidoreductase chain 4L	mt:ND4L	FBgn0013683	5.45	-2.45	4.84	0.00	0.00
CG5080	-	CG5080	FBgn0031313	5.41	-2.43	3.99	0.00	0.00
			FBgn0261451	5.28	-2.40	7.34	0.00	0.04
CG41623	Ubiquinol-cytochrome c reductase 11 kDa subunit	UQCR-11	FBgn0260008	5.21	-2.38	7.09	0.00	0.01
CG10512	-	CG10512	FBgn0037057	5.18	-2.37	6.42	0.00	0.02
CG34063	mitochondrial NADH-ubiquinone oxidoreductase chain 2	mt:ND2	FBgn0013680	4.66	-2.22	8.11	0.00	0.01
CG4869	beta-Tubulin at 97EF	betaTub97EF	FBgn0003890	4.39	-2.13	6.61	0.00	0.02
CG14032	Cyp4ac1	Cyp4ac1	FBgn0031693	4.16	-2.06	7.48	0.00	0.01
CG13628	Rpb10	Rpb10	FBgn0039218	4.03	-2.01	5.50	0.00	0.03
CG32744	Ubiquitin-5E	Ubi-p5E	FBgn0086558	3.70	-1.89	7.86	0.00	0.01
CG15717	-	CG15717	FBgn0030451	3.67	-1.88	4.82	0.00	0.02
CG6206	Lysosomal alpha-mannosidase II	LManII	FBgn0027611	3.55	-1.83	10.82	0.00	0.02
CG3618	-	CG3618	FBgn0037028	3.38	-1.76	5.29	0.00	0.04
CG11880	Choline transporter-like 2	Ctl2	FBgn0039637	-3.50	1.81	4.80	0.00	0.04
CG14489	olf186-M	olf186-M	FBgn0015522	-3.83	1.94	6.17	0.00	0.01
CG42274	Rho GTPase activating protein at 18B	RhoGAP18B	FBgn0261461	-3.85	1.95	7.09	0.00	0.03
CG4860	-	CG4860	FBgn0037999	-4.02	2.01	6.10	0.00	0.00
CG10641	Swiprosin-1	Swip-1	FBgn0032731	-4.15	2.05	6.90	0.00	0.04
CG17260	-	CG17260	FBgn0031498	-4.15	2.05	4.10	0.00	0.02
CG4178	Larval serum protein 1 beta	Lsp1beta	FBgn0002563	-4.37	2.13	7.01	0.00	0.01
CG13117	-	CG13117	FBgn0032140	-4.75	2.25	8.68	0.00	0.01
CG13608	mitochondrial ribosomal protein S24	mRpS24	FBgn0039159	-5.17	2.37	4.05	0.00	0.01
CG4680	Gag-related	Gagr	FBgn0036627	-5.23	2.39	4.12	0.00	0.04
CG5399	-	CG5399	FBgn0038353	-5.35	2.42	8.45	0.00	0.03
CG34166	-	CG34166	FBgn0085195	-5.64	2.50	5.42	0.00	0.00
CG6806	Larval serum protein 2	Lsp2	FBgn0002565	-5.66	2.50	4.61	0.00	0.00
CG11086	Growth arrest and DNA damage-inducible 45	Gadd45	FBgn0033153	-6.01	2.59	6.23	0.00	0.00
CG32495	Glutathione synthetase 2	Gss2	FBgn0052495	-6.77	2.76	3.56	0.00	0.01
CG14868	-	CG14868	FBgn0038330	-7.20	2.85	2.34	0.00	0.04
CG33126	Neural Lazarillo	NLaz	FBgn0053126	-7.51	2.91	3.69	0.00	0.03
CG12505	Activity-regulated cytoskeleton associated protein 1	Arc1	FBgn0033926	-7.71	2.95	8.14	0.00	0.00
CG10337	-	CG10337	FBgn0032805	-8.85	3.15	3.01	0.00	0.04
CG14695	-	CG14695	FBgn0037850	-8.87	3.15	4.17	0.00	0.00
CG4859	Matrix metalloproteinase 1	Mmp1	FBgn0035049	-10.02	3.33	6.56	0.00	0.04
CG31359	Heat-shock-protein-70Bb	Hsp70Bb	FBgn0013278	-10.36	3.37	2.92	0.00	0.04
CG6725	Sulfated	Sulf1	FBgn0040271	-10.62	3.41	4.59	0.00	0.00
CG15009	Ecdysone-inducible gene L2	ImpL2	FBgn0001257	-11.68	3.55	2.73	0.00	0.00
CG10076	spire	spir	FBgn0003475	-12.74	3.67	3.72	0.00	0.01
CG3694	G protein subunit gamma at 30A	Ggamma30A	FBgn0267252	-13.46	3.75	6.76	0.00	0.00
CG5847	zye	zye	FBgn0036985	-16.11	4.01	2.90	0.00	0.03
CG4408	-	CG4408	FBgn0039073	-18.99	4.25	5.55	0.00	0.00
CG34436	-	CG34436	FBgn0085465	-22.04	4.46	5.88	0.00	0.00
CG17914	yellow-b	yellow-b	FBgn0032601	-26.81	4.74	2.99	0.00	0.02
CG15678	poor Imd response upon knock-in	pirk	FBgn0034647	-50.78	5.67	4.62	0.00	0.00
CG34296	-	CG34296	FBgn0085325	-55.49	5.79	6.63	0.00	0.00
CG6124	eater	eater	FBgn0243514	-200.89	7.65	7.81	0.00	0.00

Supplementary Table 1. Genes differentially expressed between *w*^{1118} and *eater*^{1} hemocytes from L3 wandering larvae. Filtering applied: p-value < 0.05, FDR < 0.05, CPM < 5, FC > 1.88 or < -1.88. Negative FC (fold change) means lower expressed in *eater*^{1}, positive FC means higher expressed in *eater*^{1}.

Chapter 4: Conclusions

The purpose of this doctoral thesis was to provide a deeper characterization of two phagocytic receptors, Eater and NimC1, in the *Drosophila* cellular immune response.

Eater and NimC1 are two EGF-like repeat Nimrod surface receptors specifically expressed in hemocytes, the *Drosophila* blood cells (Kocks et al., 2005; Kurucz et al., 2007). Both proteins have been shown to mediate Gram-positive, but not Gram-negative, bacteria phagocytosis (Bretscher et al., 2015; Kocks et al., 2005; Kurucz et al., 2007). However, the specific involvement of NimC1 in *Drosophila* immunity has never been tested with the use of a null mutant. For this reason, we generated a null mutation in *NimC1* (called *NimC1*^{1}), by homologous recombination (Baena-Lopez et al., 2013), and re-visited its function in hemocyte-mediated immunity.

Using this genetic tool, we clarified the role of this receptor in phagocytosis by showing that NimC1 is dispensable for engulfment of Gram-positive and Gram-negative bacteria. Nevertheless, NimC1, but not Eater, is required for engulfment of latex beads and yeast zymosan particles (i.e. particles without any bacterial motifs on their surface). Interestingly, by using the *NimC1,eater* double mutant we were able to show that NimC1 does contribute to phagocytosis of both Gram-negative and Gram-positive bacteria, but its function is masked by Eater. Accordingly, *NimC1,eater* double mutant hemocytes are deficient in the phagocytosis of all types of bacteria, as well as nonimmunogenic particles. This suggests that Eater and NimC1 are the two main phagocytic receptors for bacteria in *Drosophila*. In particular, this work indicates that both receptors contribute synergistically to microbes' phagocytosis, notably in the initial step of bacteria adhesion to the hemocyte surface.

Receptor synergy during particle engagement and engulfment has been largely explored in the past, notably in mammals (Freeman and Grinstein, 2014). A synergistic collaboration between phagocytic receptors could be achieved by increasing the phagocyte affinity for the targeted particle by simultaneous recognition of different motifs by different receptors and/or co-receptors. This is the case for apoptotic cell engulfment, where MFG-E8 and oxidized phosphatidylserine can be recognized by integrins and CD36, respectively (Heit et al., 2013). Also in *Drosophila* it has been shown that *S. aureus* is recognized in a dual model by Draper and an integrin, which recognize distinct targets on the microbe surface (Shiratsuchi et al., 2012). Yet, the engagement of a phagocytic receptor can lead to an increase in the mobility of other (co-)receptors in the plasmamembrane, as in the case of integrins after FCγRs

engagement, resulting in receptor synergy (Jongstra-Bilen et al., 2003). In our case, we suggest a model of bacterial phagocytosis where the Eater and NimC1 proteins have distinct roles in microbial uptake, functioning as tethering and docking receptors, respectively. We suggest that Eater works as the key binding receptor, recognising specific motifs on the microbial surface. The strong bacteria adhesion defect observed in *eater* single mutant hemocytes when incubated with *S. aureus* bacteria is in agreement with our hypothesis. Therefore, we think that Eater binding serves to bring microbes close enough to the phagocyte surface to allow other (co)-receptors, with lower affinity, to interact and trigger the downstream internalization cascade. NimC1 would hence function in this latter event, likely as a subunit of a bigger macromolecular complex. The nature of the complex mediating the internalization signalling is unknown. One of the best characterized intracellular cascades during phagocytosis in *Drosophila* is the Draper-mediated one, notably during glial engulfment of apoptotic bodies (Ziegenfuss et al., 2008). We could speculate that Eater and NimC1 promote the downstream engulfing event via Draper signalling. Draper, as NimC1 and Eater, belongs to the Nimrod family of proteins and has been implicated in phagocytosis of bacteria (notably Gram-positive) and apoptotic cells (Hashimoto et al., 2009; Kuraishi et al., 2009; Shiratsuchi et al., 2012; Tung et al., 2013). Interestingly, preliminary data from our lab indicate that Draper protein levels are increased in *NimC1,eater* double mutant hemocytes (Petrignani et al, unpublished). This could be the result of a compensation mechanism: since the "tethering" and "docking" players are missing (i.e. Eater and NimC1, respectively), the engulfing system attempts to compensate their absence by over-expressing the responsible of the intracellular signalling. In agreement with this view, bacteria binding to *draper* mutant hemocytes was not affected, in favour of Eater being the key tethering receptor. Nevertheless, until further analysis, a role of Eater as signalling receptor cannot be completely ruled out yet. The use of truncated e*ater* mutants carrying a deletion of the cytosolic tail, in combination or not with a substitution of the transmembrane domain, will help us clarifying the role of Eater as a phagocytic receptor. Eater and NimC1 bacteria ligands are still unknown. However, it has been shown that lipoteichoic acid is required for Draper, but not NimC1 or Eater, mediated phagocytosis of *S. aureus* (Hashimoto et al., 2009), suggesting that the three receptors would recognize distinct microbe motifs. The binding of different receptors to multiple ligands on the same target would be a manner, as described above, to enhance the phagocyte affinity for the bacterium, improving therefore the engulfing event. Moreover, an additional hypothesis on Draper, NimC1 and Eater working together in phagocytosis could be advanced from an evolutionary perspective. It has been shown, indeed, that Nimrod C-type receptors (to whom Eater and NimC1 belong) evolved from Draper-like

proteins after a series of modifications in their protein structure, notably changes in their domain composition (Somogyi et al., 2008). It is possible, therefore, that Nimrod C-type members did retain some functional properties of the Draper-like proteins (such as the involvement in bacteria phagocytosis), but underwent sub-specialization, by acquiring new specific properties and functions. Finally, another way NimC1 and Eater could interact to promote bacteria engulfment is through physical association of their extracellular (and/or transmembrane) domains, improving the affinity for their cognate ligand and the subsequent activation of the internalization signal. Co-immunoprecipitation or FRET experiments can be performed in future studies to understand whether a close physical interaction between those two receptors exists during phagocytosis. Interestingly, preliminary data non presented in this thesis, revealed a synergistic effect of NimC1 and Eater also in regards to apoptotic cells engulfment (upon *ex vivo* apoptotic bodies phagocytosis assay). This suggest that the role of these proteins would go beyond a pathogen-related function, also playing a role in the tissue homeostasis.

In addition to NimC1 and Eater contribution to phagocytosis, this work uncovered a novel role of these receptors in hemocyte adhesion, proliferation, and survival. In third instar larvae, indeed, those receptors negatively regulate hemocyte counts in an additive manner, and function as adhesion molecules, by controlling hemocyte spreading. Moreover, we showed that, in contrast to Eater, NimC1 is not directly required for hemocyte sessility *in vivo*, both during larval stages and adulthood (data not shown). Interestingly, although increased in third instar larvae, hemocyte number in *eater*^{1} young adults is wild-type like, and declines significantly faster with age compared to control flies. This last observation raised two interesting issues. Firstly, a mechanism of hemocyte number compensation might exist in *Drosophila*, to avoid having excessive hemocytes number during adulthood. This is in agreement with a recent study showing that aberrant hemocytes number is deleterious for the host under specific conditions (Ramond et al., 2019). This would hence imply an ability of the lymph gland to sense peripheric proliferation adjusting the number of mature hemocytes production, therefore suggesting a feedback between peripheric and lymph gland hematopoiesis. In line with this hypothesis, percentage counting of *HmlΔdsred.nls* cells in *eater*^{1} lymph gland of L3 wandering larvae resulted slightly decreased compared to wild-type control, although not statistically significant. It will be worth to analyse hemocyte counts in young adults of other mutant backgrounds causing an increase in larval peripheric blood cell number, to further confirm this hypothesis. Secondly, the observation that *eater*^{1} adults possess less hemocytes compared to wild-type suggests that blood cell sessility might be an important

requirement for their survival, specifically during adulthood. Why would not this be the case also for larval stages? The physiological challenges that an adult hemocyte receives are different from those of a larval one. Therefore, we could assume that the pathways regulating the hemocyte life and death decision change according to the developmental stage of the animal, reflecting the physiological stresses that the cell type might encounter. For example, larval hemocytes undergo disruption of their sessile state after immune challenge by the parasitic wasp *L. boulardi* (Zettervall et al., 2004). After wasp egg deposition into the larval hemocoel, indeed, the subepidermal sessile blood cells are detached and released into circulation where they differentiate into lamellocytes (Markus et al., 2009). Yet, an ecdysone pulse at the onset of pupariation is responsible of hemocytes dispersal during metamorphosis, during which larval tissues are remodelled and destructed by the mobilized hemocytes (Regan et al., 2013). To conclude, it is possible that adult hemocytes only specifically require sessility to receive survival signals coming from their attachment to the extracellular matrix (ECM). It is well known, indeed, that after a cell dissociate from its ECM substratum undergoes programmed cell death, also known as anoikis (Paoli et al., 2013).

An intriguing question that deserves future investigations is how NimC1 and Eater functionally regulate cell adhesion, proliferation, and survival. In particular, are they working as signalling receptors? And yet, is each pathway individually regulated, or is one the consequence of the other? To attempt answering these questions, we performed RNA-seq on naïve *eater* mutant and *wild-type* larval hemocytes. Our preliminary functional analysis did not bring any evidence of Eater being a signalling receptor. On the contrary, we rather believe that this Nimrod protein serves as an adhesion molecule, anchoring the hemocyte to its ECM substratum. How is the cell able hence to signal, through Eater, an adhesion response? In particular, is Eater-dependent phagocytosis and adhesion relying on the same intracellular signalling mechanism? The integrin receptors represent a family of well characterized signalling molecules regulating cell adhesion and shape, proliferation, and survival (Stupack and Cheresh, 2002). In addition, integrins often cooperate with other receptors to fully induce an intracellular signalling (Stupack and Cheresh, 2002). We could therefore speculate that this receptor family collaborates with Eater to trigger an adhesion response, that might influence, as a consequence, the cell proliferation behaviour of the hemocyte. In *Drosophila*, the integrin family comprises five α and two ß subunits. It has been shown that *Drosophila* integrins control hemocyte shape and embryonic migration during early development (Huelsmann et al., 2006; Siekhaus et al., 2010). Moreover, by mediating cell adhesion to the ECM, integrins often regulate the intracellular pathways required for cell survival (Stupack and Cheresh, 2002). Therefore, they

might cooperate with Eater during adulthood to convey the required life and death cell decision. Finally, integrin contribution for signalling Eater-dependent phagocytosis cannot be excluded yet. However, *draper* mutant larvae do not show hemocytes sessility defect (data not shown), suggesting that this receptor might not be involved in the regulation of blood cell sessility.

To conclude, this study provided new insights on Eater and NimC1 as the two main receptors for phagocytosis of bacteria in *Drosophila,* with each receptor likely playing a distinct role in microbial uptake. In addition, this body of work uncovered a potential role for cell adhesion in regulating hemocyte proliferation, as well as hemocyte survival during adulthood.

Deciphering the signalling mechanisms between these surface receptors and the regulatory proteins involved in cytoskeleton modifications (which are required for both phagocytosis and cell adhesion) represents one of the future challenges to further understand *Drosophila* cellular immunity.

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Curriculum Vitae

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- Education

2015 – present PhD in Molecular Life Sciences, École Polytechnique Fédérale de Lausanne, Switzerland

2012 – 2014 MSc in Molecular Biology, University of Padova, Italy
Final mark: 110/110 cum laude

2009 – 2012 BSc in Molecular Biology, University of Padova, Italy
Final mark: 103/110

1999 – 2009 Piano degree, Conservatorio di Musica "C. Pollini", Padova, Italy
Final mark: 9/10

- Research experience

2017 – present PhD student
Global Health Institute, EPFL, Lausanne, Switzerland
Projects: "Role of the Nimrod proteins in phagocytosis in *Drosophila melanogaster*"
Supervisor: Prof. Bruno Lemaitre

2015 – 2017 PhD student
Swiss Institute for Experimental Cancer research, EPFL, Lausanne, Switzerland
Projects: "Analysis of SIN regulation by Byr4p and Etd1p in *S. pombe*"
Supervisors: Prof. Viesturs Simanis

2013 – 2014 Master thesis project
Department of Cellular Biology, University of Geneva, Switzerland
Project: "PHO84 gene expression analysis at a single cell level in co-suppression condition in *Saccharomyces cerevisiae*".
Supervisor: Prof. Françoise Stutz

January-July 2012 Bachelor thesis project
Department of Biology, University of Padova, Italy
Project: "Role of miR-27a and miR-144 in the response of A549 human cells to gamma radiation".
Supervisor: Dott. Maddalena Mognato

- Teaching experience

2015 – 2016	**Practical work in Life Sciences I**, to EPFL Bachelor students
2015 – 2016	**Practical work in Life Sciences II**, to EPFL Bachelor students
2015 – 2016	**Physiology lab I**, to EPFL Bachelor students
2015 – 2016	**Physiology lab II**, to EPFL Bachelor students
2016	**Physiology by systems II**, to EPFL Bachelor students

- Supervision experience

July-October 2018 Supervision of a Medical Student from Kyoto University

- Conference oral presentations and posters

2018 *Swiss Drosophila Meeting*, Fribourg, Switzerland, oral presentation
2018 *EMBO Workshop on Phagocytosis of dying cells*, Ghent, Belgium, poster
2019 *60th Annual Drosophila Research Conference*, Dallas, Texas, USA, oral presentation
2019 *26th European Drosophila Research Conference*, Lausanne, Switzerland, poster

- Publications

"Two Nimrod receptors, NimC1 and Eater, synergistically contribute to bacterial phagocytosis in *Drosophila melanogaster*" (Research article)
C. Melcarne, E. Ramond, J. Dudzic, A. Bretscher, E. Kurucz, I. Ándo, B. Lemaitre
2019, ***The FEBS Journal***

"Phagocytosis in *Drosophila*: from molecules and cellular machinery to physiology" (Review)
C. Melcarne, B. Lemaitre, E. Kurant
2019, ***Insect Biochemistry and Molecular Biology***

- Qualification courses

-*Introduction to Cancer Genomics*, Université de Lausanne, Switzerland
-*Scientific writing for biomedical articles*, EPFL, Switzerland
-*The making of an innovative medicine*, EPFL, Switzerland

- Extra activities

2008/2009	Student representative in the Council of administration.
2012/2013	Student representative at the University of Padova.
2016-2018	Committee member of the Joint EPFL-UNIL PhD Retreat (organization of 2 days retreat for 60 PhD students to share their work and to create new network opportunities).
2015-2018	Committee member of the Science de la vie EPFL PhD Hiring days.